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Simultaneous, single-carrier delivery of antigens and immune-modulatory molecules to dendritic cells

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Simultaneous, single-carrier delivery of antigens and immune-modulatory molecules to dendritic cells

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2013

Dedication

I would like to dedicate this dissertation and my Ph.D. work to my family. They have instilled upon me an interest and drive to pursue science throughout my entire life. Without their love and support I would not be the person that I am today.

Acknowledgements

I would like to thank, first and foremost Dr. Krishnendu Roy for allowing me to work in his laboratory for the last seven years. I have learned a lot from him and from my experiences here. If it were not for him, I honestly would have quit seven years ago. I appreciate that he saw something in me that was worth fighting for (and more frequently than not, fighting with). I thank you for the opportunity to work in your group and learn from you and the other students these past few years. I would next like to thank Dr. Nicholas Peppas for taking me on as a co-advisor. I honestly can say, especially at that particular point in my Ph.D. studies, it was so amazing to be taken into another lab with such open arms. I truly feel honored to have been able to be a part of your research group and learned a lot from you and your students. The interest you take in your students and the passion for science that you encourage and support is truly impressive. I thank you for giving me the opportunity to be a member of your group and for all of the other opportunities that you have offered me. I would also like to thank the rest of my committee, Dr. Laura Suggs, Dr. Jennifer Maynard, and Dr. McConnville for your guidance.

I would like to thank Dr. Laxminarayana Raja in Aerospace Engineering for the opportunity to work with his plasma glow discharge technology. I would like to thank Dr. Jonathan Sessler and Dr. John DiGiovanni, the faculty advisors for the Cancer Prevention Research Institute of Texas (CPRIT) graduate trainee program. Their input and questions during our bi-weekly CPRIT seminars are always interesting and insightful. I have been fortunate enough to have the help of a large number of really great scientists at the core facilities here at the University of Texas including: Hugo

Celio, Damon Smith, Dwight Romanovicz, Angela Bardo, and Julie Hayes. The University of Texas has one of the greatest research centers in the country and the ability to have access to the equipment that they maintain and train the students to use is an invaluable resource. I would like to thank Jennifer Cassaday and all of the other employees at the Animal Resource Center. I appreciate the difficult nature of their jobs and have the utmost respect and admire them for the way that they conduct themselves.

I would like to thank my lab mates in Dr. Roy's lab. It strikes me as I am completing my studies here how close I have become to this group of extraordinary people and how hard it is to be leaving them. In an odd way, the people that I work with have become a crazy, mostly dysfunctional, family. We've fought like siblings and warred through family feuds but share a bond that cannot be broken, not to mention have a closeness that make other labs visibly uncomfortable in public settings. I can only assume that they are jealous of us. Klub Krish has become an exclusive group and I wouldn't trade my membership in it for anything in the world. I would like to thank all of the current and former members of Klub Krish for their help, support, guidance, and friendship. I would like to especially thank Krista Fridley, probably the only person in the lab that was willing to put up with the mess that is the way I tend to keep the office. She was most frequently my partner in crime in lab, the first person that I knew I could turn to for help with something, and, more importantly, the main person who I knew, regardless of how ridiculous I was being, would join me in any angry rant on any subject. I would like to thank Lonnissa Nguyen. Lonnissa is a fierce person, in every way, but most especially in the way she cares about people. She is one of the most passionate people that I have ever met and is an amazingly hard worker. I would like to thank Ankur Singh, a good friend and an amazing scientist. There was a point during my studies that I had convinced myself that, sometimes, just talking about an experiment in

Ankur's presence would guide me towards the right design. I would like to also thank Ankur for allowing me to help get him into trouble occasionally. While I do not take full blame for a lot of the terrible things I introduced him to (walking like a thug, drinking Long Island Iced Teas, etc.), I would like to think that I enriched some of his first few experiences in the United States. I would like to thank Sabia Abidi, my evil birthday twin and the co-inventor of Klub Krish. She made my first few months at UT in the lab more fun than I should probably admit. Lab late at night transformed into an awkward dance club party capable of rivaling any bar in downtown Austin (though Hui, who caught us one night, would probably have argued otherwise given our dance skills). I would like to thank Jian Lin for his input while I was having a hard time in the lab with my work. He also has been an incredibly supportive friend since leaving the laboratory. Jian really embraces life in, oftentimes, unexpected ways and for that I appreciate having him around. I would like to thank Jardin Leleux for being a great person to work with on my final Ph.D. project. I cannot think of someone better to be working with right now. She has a positive attitude, drive, sincere interest in science and just exudes a certain level of optimism and positive way of thinking that I think is so hard to loose when you've been in graduate school for a long time. I find her attitude admirable and something to really push myself towards (or back to) as I've been completing my work here. I would like to thank Irina Fernandez for all of her professional help and advice. She has such a tremendously positive attitude and has such a fantastic way of being able to acknowledge a problem and just decide to deal with it. I would like to thank Prinda Wanakule, Tracy Ooi, and Asha Fleury for being such good friends to me, especially during a very difficult time in my life. When I was at a very low point they were there to try to pick me up, even if I wanted to sit and wallow in my bad mood. They always bring a sense of fun and adventure to things that I find trouble putting into words. Prinda,

hands down, has shared some of the funniest conference/travel experiences that I imagine I will ever have. She will definitely be remembered as being one of my favorite travel companions. While I am tempted to explain this further, I fear that it would take more than a paragraph to explain, so I'll assume that if she looks at this she'll understand why I didn't delve further into it. Tracy, with her infectious laugh, never ceases to surprise me with her sense of humor about things. I can't count the number of times that I have been upset about something and she has looked at me angrily and made a joke about doing something slightly inappropriate to the person I'm angry at (I must emphasize this is done as a joke) and it just snaps me out of whatever bad mood I was in. Asha, though I've known her the least amount of time, has proven to be a great person and an excellent co-conspirator especially when it comes to winning awesome ramp building competitions. I would like to thank Rachit Agarwal (a.k.a Chacha Racha or a.k.a. Rachito depending on my mood) for being the annoying little brother I never knew that I wanted. Rachit, if you ever figure out what I stole from your lab bench I will pay you $100. Also, while I may be credited with teaching Chacha how to play hooky from lab, I would say that I am hardly the master of this. This was, however, a fun tradition of mine and Rachit's where during spring break we would leave lab early one day and see what was going on during South by Southwest. I would like to thank Michelle Kim for acting as a calming force in the lab. I would like to thank Pallab Pradhan for all of his help with my *in vivo* studies. Pallab has this amazing laugh that can somehow make even the worst of lab situations not seem so bad. I would like to thank Gazell Call who has offered me such kindness and understanding and honestly, really offered to take care of me when she really didn't have to or have a reason to be so kind. I would like to thank Bilal Ghosn for his sound scientific advice and for really making graduate school an interesting experience. I would also like to express my sincere gratitude to Michelle Miller, an honorary member

of Klub Krish, for the sheer number of times she has saved me, either by putting in a last minute rush order for me or for providing me with chocolate when I was in a bad mood. Last, but most certainly not least, I would like to thank Mary Moore. I cannot count the number of times that Mary has helped me navigate a professional (or personal) situation that I most certainly would have awkwardly ruined. This isn't to say that she was capable of preventing some of my more awkward social situations, but she was also always there to try to convince me that whatever I had done wasn't as bad as I thought, or laugh with me when it really was. She has always been a very helpful lab mate, and I cannot begin to describe how lucky I personally am that she stayed at UT to complete a post-doctoral studies. Klub Krish, it's a lifestyle.

I have had the fortune of working with a number of undergraduates while I was here, but only one was really capable of handling my work hours and obsessiveness about technique: Tiffany Vo. Tiffany was an incredibly hard worker, but more than that she was also incredibly loyal and trustworthy. During all of those long hours working (she worked with me not only on my dissertation research but also on a stem cell based project) she truly became a friend. I am so excited that she is doing so well in graduate school and cannot begin to describe how happy I am for all of her successes.

I would like to thank all members of the Peppas lab. I have not been in the lab as long to have quite as many personal experiences with the lab, but they have really made an effort to make me feel like a member of their group, and for that I am grateful. I would like to especially thank Brandon Slaughter. I have really appreciated having the fortune of being able to consider him a friend and cannot express how much he has helped me, especially this past year. I would like to thank William Liechty. Bill's interest in science and enthusiasm for work is something that I find extremely admirable. I would like to thank Amey Puranik who I had the fortune of working on a review paper

with. He is an incredibly hard worker who was incredibly patient with me and understood the nature of the work that I was doing often resulted in my having an awkward work schedule.

I would like to thank all of the friends that I have been fortunate enough to make during my time here. I would especially like to thank the girls that got me through my first few years of graduate school: Kathryn Erickson, Justina Tam, Krista Fridley, and Katy Moncivais. We had become so inseparable that there was a running joke that we had become a sorority. I cannot imagine making it through my first two years of graduate school without this group of women. I would like to especially thank Katy Moncivais, my awkward partner in crime. To give Katy the kind of acknowledgement that she would deserve would be several pages long and probably admit many horrible things about ourselves and the closeness of our relationship that would make any reader uncomfortable. Katy has truly become more than a friend to me, she is family. I would also like to take a moment to thank Katy's family for adopting me as one of their own here in Texas. I would like to take a moment to especially thank the following friends for their support and also for their help and attention to my work even though they work in different fields: Gayle Berstein, Tom Linskey, Adrien Ponticorovo, and Arnold Estrada. Their input in my work was invaluable to me and I appreciate the time that they gave me. I would similarly like to thank the Baker Boys: Peter Voyvodic, Anthony Monteforte, Subhamoy Das, and Jason Lee. I would also like to thank the following friends for their constant love and support: Naznin Daginawala, Wakeem Abraham, Rashad Reddick, Nancy Serafino, Su Jung Hong, Jung Chang, Arun Honda, Kevin Nealis, David Lin, Rafael Feliciano, Joo Kim, Nayyereh Rajaei, and Umar Hussain. These are a group of people who I feel honored to consider friends. I know that regardless of where my life

takes me this group of people will always be on my side and ready for a fight (though as we get older this probably should be more of a theoretical fight).

I would also like to take a moment to thank two professors from Rutgers University who inspired me to further my studies: Dr. Prabhas Moghe and Dr. Kathryn Uhrich. I worked in Dr. Moghe's lab for three years while I was an undergraduate. The support, guidance, and, ultimately, faith that he had in me when I was a young researcher really molded me into a scientist and gave me confidence in my abilities. I would like to thank Dr. Uhrich for all of her support and invaluable guidance that she supplied outside of the laboratory. In a field that tends to be dominated by men, I cannot begin to describe to you how inspiring of a person she is and how grateful I am to have had her advice while I was in college. She really is a great role model and I feel truly honored to have been able to learn from her. During my time at Rutgers I worked with many fantastic people in the lab, but would like to single out a few in particular: Nicole Plourde, Evangelia Chnari, Ram Sharma, and Jinzhong Wang. As an undergraduate, these graduate students truly provided me with so much guidance and honestly, I learned so much from them I really can't begin to describe how thankful I am for having had them as mentors as an undergraduate research assistant.

Lastly I would like to thank my family. I would like to thank my dad for giving me the inspiration for studying biomedical engineering. Many years ago, when I first heard about biomedical engineering as a field, I was told that scientists study problems while engineers fix them. From that moment on it was a dream of mine to work towards helping people because, truthfully, I always admired all of the new innovations and medicines that my dad was fortunate enough to receive. I joke around about how my dad put his foot down when I was growing up and provided me with an ultimatum of either working as a summer camp counselor or going off to "nerd camp" for the summer instead

of sitting at home listening to punk rock music and heading to the village in New York City. If it were not for him doing this, I have no doubt that I would not have had all of the opportunities that I have had in science. I would like to thank my mother who has given me the stubborn will to push myself through the Ph.D. program, even when I thought about giving up. She constantly has been an inspiration to me even when just reminding me to focus and keep putting one foot in front of the other and always push forward. I would like to thank my brother for constantly reminding me to find the humor in any given situation. People that know me fairly well know that I am almost always looking for an excuse to laugh at any given situation and try to focus on the humor of any given situation. He constantly reminds me to look for the positive in life and can make me laugh regardless of what's going on.

Simultaneous, single-carrier delivery of antigens and immune-modulatory molecules to dendritic cells

Eileen Regina Dawson, Ph.D.

The University of Texas at Austin, 2013

Supervisors: Krishnendu Roy and Nicholas Peppas

Immunotherapy as a means for cancer treatment has been investigated for over a century. While studies have been completed using different immunological strategies, development of a clinical therapeutic cancer vaccine has proven elusive. Recently, success has been seen with prophylactic vaccines for cancers with known viral origins (Gardasil® and Cervarix for Human Papiloma Virus). However, such strategies do not address the challenge in generating effective immune response against other tumor antigens, most of which are weakly immunogenic self-antigens. Tolerance to these self-antigens could ultimately limit the patient's ability to mount an effective anti-tumor immune response.

The US Food and Drug Administration recently approved the first DC cell-based cancer vaccine, Provenge®, for use in prostate cancer. This vaccine requires cell isolations from the patient as well as *in vitro* DC modifications, which ultimately leads to high cost as well as multiple procedures. However, results indicate that, on average, patients live only four months longer than those receiving a placebo. While this work remains important, and offers proof that priming DCs can improve the lifespan of a patient, it ultimately does not offer a long-term cure. Direct and highly efficient *in vivo*

delivery of antigens to DCs could overcome the challenges associated with *ex vivo* DC manipulation and may offer a more scalable method for generating anti-tumor immunity.

This research focuses on the development of novel formulations that allow simultaneous delivery of protein/peptide-based tumor antigens and immune-modulatory nucleic acids (siRNA and immune stimulatory CpG) to the same dendritic cells (DCs) *in-vivo*. Such formulations allow a synthetic immune-priming center to be created at the site of immunization and simultaneously deliver the tumor antigen to DCs and modulate their immune response through IL-10 silencing. Our hypothesis is that using such a DC-targeted dual delivery system we will be able to illicit strong T helper 1 (T_H1) and Cytotxic T Lymphocyte (CTL) response *in vivo* against a wide array of tumor antigens. This can become a platform technology where the biomolecules (antigen and immunomodulatory agents) can be easily varied based on particular cancers.

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CHAPTER 1

Introduction: Specific Aims and Overview

1.1 INTRODUCTION:

Immunotherapy as applied to infectious disease is considered to be the most cost-effective and safe method for the control of many diseases. Immunization has had a tremendous impact on health worldwide, as demonstrated by the global eradication of smallpox: there has not been a single reported case since 1977, however, new immunization strategies need to be employed in order to generate effective immune responses to chronic viral or tumor antigens, most of which are self-antigens (Friede and Aguado, 2005). While many of the most potent vaccines on today's market are given as a live attenuated or a killed form of the microorganism, many potential drawbacks exist for both of these strategies. These include poor efficacy of boosting antibody responses and safety concerns. For example, the pertussis attenuated bacterial vaccine has been shown to cause seizures, encephalitis, brain damage, and even death. By applying recent immunological findings such as new information about the cells of the immune system and the microenvironments within which these cells reside to vaccine development, it may be possible to create a safer, more cost effective vaccination platform capable of eliciting the appropriate immune response specific for the disease (Hubbell et al., 2009). My thesis work is focused on doing that by applying a biomaterial-based approach to create a protein-based vaccine delivery platform capable of generating both an antigen specific immune response as well as one that is T helper (T_H) 1 specific and capable of eliciting a cytotoxic T-cell (CTL) response (Kasturi et al., 2005; Singh et al., 2008, 2009). Previously, our laboratory has completed work on creating a biomaterial-based dendritic cell priming center in order to attract immature DCs to the location of microparticle

administration in order to more effectively target DCs for transfection using a DNA based vaccine (Singh et al., 2009). Although our results indicate that this is a promising new immunization option, the overall potency of DNA vaccines in human trials is low and can be attributed to low efficiency of DNA transfection to translation into antigenic proteins. This is further affected by inefficient presentation of translated proteins on surface of antigen presenting cells. Here we hypothesize that by delivering the actual protein antigen peptide (by-passing the need for cellular transfection) we will be able to induce T_H1 specific responses to our antigenic protein more effectively.

Most viral formulations function by targeting antigen presenting cells (APCs) such as B cells, macrophages and dendritic cells (DCs), which are known to be the most prominent T-cell activators. Although dendritic cells have the potential to act as a cellular vaccine, clinical trials of ex-vivo major histocompatibility complex (MHC) primed DCs have shown a lack of persistent expression of MHC Class I complexes on their surface. These complexes only remain on the surface on the order of a few hours making long term immunity using these methods improbable. These procedures would also require cell isolations as well as in vitro DC modifications, which would ultimately lead to high cost as well as additional procedures for the patient. Additionally, and more importantly, 90% of these transplanted DCs die and very few of the surviving cells actually migrate to the lymph node (Ali et al., 2009). Therefore, in vivo protein delivery to DCs would be preferential. Bolus injections of proteins are, however, considerably less immunogenic when administered alone and are frequently subject to degradation and clearance from the body. One way to enhance protein uptake by APCs is by delivering them via particulate systems. Because particulate delivery systems are more comparable in size to common pathogens (~ 1 um) APCs have been shown to preferentially uptake these particles and therefore the particle associated proteins as well. This enhanced uptake by APCs is an

important contributing factor in the ability of these delivery systems to induce more potent immune responses than soluble antigens. Additionally, by using a particulate system, it would be possible to ensure delivery of more than just a single protein molecule as well as combine delivery with an adjuvant or other immunomodulatory molecules (Singh et al., 2008, 2004a; Vajdy et al., 2004).

An imbalance between the T_H1 and T_H2 response has been implicated in several chronic infectious diseases such as human immunodeficiency virus infection and chronic hepatitis B and C infections. Mature T helper cells, also referred to as CD4+ T cells, are central to the induction of anti-viral responses, have been subdivided according to two predominant cytokine secretion profiles. T_H1 cells produce cytokines such as interleukin (IL)-2, interferon-gamma (IFN-γ), and tumor necrosis factor beta which are important factors responsible for promoting the cell-mediated immune response. T_H2 cells produce cytokines such as IL-4, IL-5 and IL-10 which mediate the humoral, or antibody mediated response. Cytokines released by one type of T_H lymphocyte population can down-regulate the functions of the other T_H population subset. Furthermore, it has been suggested that the initial development of APCs into T_H1 or T_H2 phenotype is believed to depend on the leading cytokines at the site of initial antigen presentation, the type of antigen presenting cell, the nature of the co-stimulatory molecules involved, and the dose of stimulatory antigens. This phenotypic commitment may be critical for the subsequent differentiation of immune cells nonspecifically recruited at the site of infection (Thompson, 1995).

The main objective of this research project is to overcome the barriers in current vaccine formulations by: a) carefully investigating different surface modification techniques to impart anionic or cationic charges to poly(lactic-co-glycolic acid) (PLGA) microparticles, b) systematically altering protein loading conditions to ensure optimal

protein dosage to DCs, and c) co-delivery of protein antigens as well as immunomodulatory molecules such as siRNA to ensure optimal CTL response. We hypothesize that using this microparticle formulation we will be able to illicit a CTL response in vivo capable of combating chronic viral diseases such as Hepatitis B and cancer.

1.2 SPECIFIC AIMS:

1.2.1 Aim 1: Optimize and characterize biodegradable, surface-functionalized, polymer microparticles for loading of proteins for the delivery to antigen presenting cells.

We hypothesize that we can impart a significant amount of cationic or anionic charge on our PLGA microparticles and that we will be able to use that charge to adsorb proteins onto the surface of the charged microparticles. The cationic charge will be achieved by functionalizing our microparticles with branched PEI as previously described (Kasturi et al., 2005; Singh et al., 2008, 2009). An anioinic charge will be imparted on our microparticles by exposing the particles to either helium or oxygen plasma using both low pressure plasma systems as well as an atmospheric plasma system developed by Dr. Raja in the Department of Mechanical Engineering at the University of Texas at Austin (Shin and Raja, 2003). Charge modification will be confirmed using zeta potential analysis. In the case of plasma treated microparticles, optimized protein loading condition experiments will be of the utmost importance as plasma surface modification may also increase several other factors altering protein adsorption most importantly: hydrophobicity and surface roughness (Fortunati et al., 2013; Shen et al., 2008). Once surface properties are analyzed we will adsorb protein electrostatically to the surface of these particles. This will be done by utilizing the protein's isoelectric point by varying key parameters to drive favorable electrostatic interactions to obtain high surface loading

of proteins, these parameters include: pH, ionic strength, and type of buffer. An overview of the project can be seen in **Figure 1.1**.

1.2.2 Aim 2: Optimize particle formulation for maximum co-delivery of immunomodulatory molecules.

Because proteins are weakly immunogenic on their own, and tolerance to our protein antigen is of the utmost concern, we will include in the formulation process an immunomudulatory molecule to enhance the immune response. We hypothesize that by utilizing electrostatic interactions we can drive protein attachment in combination with immunomudulatory molecule attachment in order to be able to deliver multiple types of molecules on a single particle. We will then evaluate the efficiency of our co-delivery system as compared to single loaded particle controls in activating DCs *in vitro* in order to mount an immune reaction. This will be done by first, looking at DC cellular activation markers using flow cytometry. We will then further study our particles ability to activate DCs by looking at both cytokine release as well as altered gene expression. Using these *in vitro* characterization methods we will choose appropriate particulate systems to move forward with for *in vivo* studies.

1.2.3 Aim 3: Test our optimized system from Aim 2 in an in vivo tumor model.

We hypothesize that our *in vitro* characterized molecules will be able to effectively limit tumor growth in an *in vivo* model. This will be done using an ovalbumin expressing melanoma cell model, murine B16 cells that were generously provided by Dr. Zhengrong Cui. We will test our formulations in both a prophylactic and therapeutic model monitoring tumor growth and survival. This will be done according to protocols

approved by the University of Texas at Austin Institutional Animal Care and Use Committee. Animals will be monitored very carefully and sacrificed at appropriate times.

1.3 OVERVIEW

Chapter 2 will focus on the background and significance of this thesis work focusing on the latest research in immunotherapy based fields. The following chapter, **Chapter 3** will focus on plasma surface functionalization of the PLGA microparticles and protein loading. It will describe the characterization in terms of surface charge, morphology, and the ability to adsorb protein to the surface of these particles. **Chapter 4** will discuss the characterization techniques of the PEI functionalized microparticles. Again, characterization will be discussed in terms of charge and ability to adsorb protein and immunomodulatory molecules to the surface. **Chapter 5** will focus on *in vitro* and *in vivo* studies to determine the most effective vaccine formulations developed in **Chapter 4**. *In vitro* characterization will be done using flow cytometry surface analysis of DC activation, cytokine secretion, and gene expression. *In vivo* studies were completed performing both a therapeutic and prophylactic model. **Chapter 6** will conclude the dissertation and discuss possible future recommendations and applications of the vaccine platform delivery system developed.

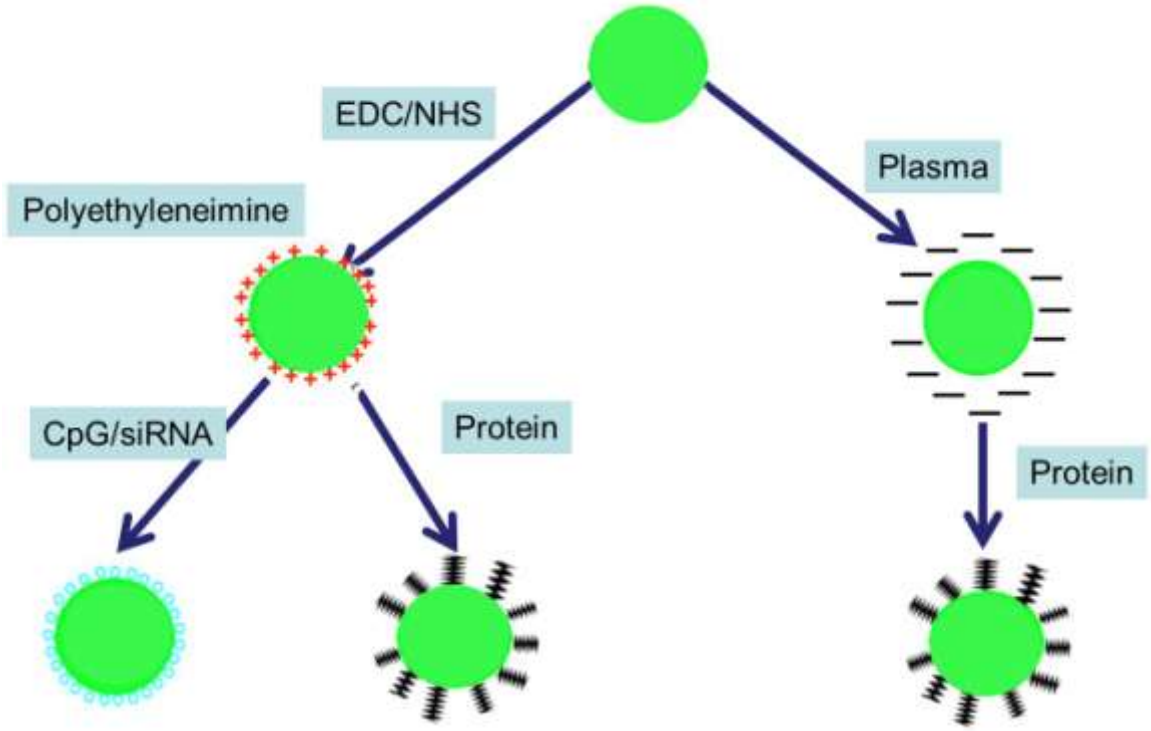

Figure 1.1 Pictorial representation of charge functionalization of particles and planned loading targets.

1.4 REFERENCES

Ali, O.A., Huebsch, N., Cao, L., Dranoff, G., and Mooney, D.J. (2009). Infection-mimicking materials to program dendritic cells in situ. Nature Materials 8, 151–158.

Fortunati, E., Mattioli, S., Visai, L., Imbriani, M., Fierro, J.L.G., Kenny, J.M., and Armentano, I. (2013). Combined Effects of Ag Nanoparticles and Oxygen Plasma Treatment on PLGA Morphological, Chemical, and Antibacterial Properties. Biomacromolecules.

Friede, M., and Aguado, M.T. (2005). Need for new vaccine formulations and potential of particulate antigen and DNA delivery systems. Adv. Drug Deliv. Rev. 57, 325–331.

Hubbell, J.A., Thomas, S.N., and Swartz, M.A. (2009). Materials engineering for immunomodulation. Nature 462, 449–460.

Kasturi, S.P., Sachaphibulkij, K., and Roy, K. (2005). Covalent conjugation of polyethyleneimine on biodegradable microparticles for delivery of plasmid DNA vaccines. Biomaterials 26, 6375–6385.

Shen, H., Hu, X., Bei, J., and Wang, S. (2008). The immobilization of basic fibroblast growth factor on plasma-treated poly(lactide-co-glycolide). Biomaterials 29, 2388–2399.

Shin, J., and Raja, L.L. (2003). Dynamics of pulse phenomena in helium dielectric-barrier atmospheric-pressure glow discharges. Journal of Applied Physics 94, 7408–7415.

Singh, A., Nie, H., Ghosn, B., Qin, H., Kwak, L.W., and Roy, K. (2008). Efficient Modulation of T-cell Response by Dual-mode, Single-carrier Delivery of Cytokine-targeted siRNA and DNA Vaccine to Antigen-presenting Cells. Molecular Therapy 16, 2011–2021.

Singh, A., Suri, S., and Roy, K. (2009). In-situ crosslinking hydrogels for combinatorial delivery of chemokines and siRNA-DNA carrying microparticles to dendritic cells. Biomaterials 30, 5187.

Singh, M., Kazzaz, J., Chesko, J., Soenawan, E., Ugozzoli, M., Giuliani, M., Pizza, M., Rappouli, R., and O'Hagan, D.T. (2004). Anionic microparticles are a potent delivery system for recombinant antigens from Neisseria meningitidis serotype B. Journal of Pharmaceutical Sciences 93, 273–282.

Thompson, C.B. (1995). Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation? Cell 81, 979–982.

Vajdy, M., Srivastava, I., Polo, J., Donnelly, J., O'Hagan, D., and Singh, M. (2004). Mucosal adjuvants and delivery systems for protein-, DNA- and RNA-based vaccines. Immunology and Cell Biology 82, 617–627.

CHAPTER 2

Background and Significance

2.1 Immunization and Dendritic Cell Function Overview

Protein antigens offer promising new treatments to diseases for which there are no current vaccines available or ones where immunity resulting from infection may not be ideal or possible in the case of cancer. Recently more than 170 antigenic peptides from 60 human tumor antigens have been discovered (van der Bruggen et al., 2013). Synthetically derived peptide sequences offer the opportunity to design a particular epitope that can be used in vaccines to mount a specific and desirable immune response (Arnon and Horwitz, 1992; Ben-Yedidia and Arnon, 1997). Although protein based vaccines are considered to be safer than traditional viral formulations (a list of current protein based vaccines can be found in **Table 2.1**) in that they are chemically defined and do not contain any potentially infectious materials, they are considerably less immunogenic when administered alone and are frequently subject to degradation and clearance from the body (Bharali et al., 2008). Therefore, most protein vaccine development strategies work on targeting specific immune cells and actively manipulating the complicated DC response in order to generate a desired immune response as reviewed by Hubbell et al. (Hubbell et al., 2009). Because protein based vaccines are non-immunogenic in order for them to be affective the proteins need to be administered in combination with another immune-stimulating material, this can be an immune adjuvant or an immune-stimulating delivery system (Leleux and Roy, 2013a).

Briefly, immature DCs take up antigens from the extracellular fluid, process them and present them on class II major histocompatibility complex (MHC) molecules. CD4+ T cells can recognize the antigen alone or with co-stimulatory molecules (these include

CD80/B7.1 and CD86/B7.2) depending on the activation state. If the protein is endogenously made, the DC will process the protein via a separate pathway and present them via class I MHC molecules, which interact with CD8+ T cells. Assuming they do not encounter a secondary pathogenic molecule, or adjuvant, they will continue to remain in the immature phenotype. DCs maturation can be induced by a variety of different signals including: pathogen-associated molecular patterns (such as LPS), bacterial DNA, double-stranded RNA, and T cell-derived signals (Banchereau et al., 2000). One of the main mechanisms that DCs use to recognize pathogenic microorganisms is the Toll-like receptors (TLRs) which can recognize specific molecular patterns present on a microbe, also known as pathogen-associated molecular patterns (PAMPs) (as reviewed in (Akira and Takeda, 2004)). The differences in DC function after maturation are illustrated in **Figure 2.1**. Only mature, activated DCs will migrate to draining lymph vessels to the nearest lymph node, while immature DCs will continue to circulate through the peripheral tissues systemically. Further, activated DCs present MHC-peptide complexes for days to ensure T cell interaction, whereas immature DC expression of MHC molecules is more transient (Cella et al., 1997; Pierre et al., 1997). If DCs do not achieve the necessary secondary activation or if weak antigens or low antigen concentrations are presented to the DCs, the DC may remain in a semi-mature state and induce immunotolerance against the presented antigen (Aichele et al., 1995; Mahnke et al., 2002; Rutella et al., 2006). Mature DCs are capable of presenting both the antigen and co-stimulatory molecules capable of activating T cells.

The type of immune response initiated at this point is dependent on a number of factors, first and foremost is the type of MHC molecule which the antigen is presented on (as mentioned previously). Another contributing factor is the cytokines present in the extracellular environment. These molecules can induce either a T_H1 or T_H2 specific

response in CD4+ T cells (as reviewed in (Banchereau and Steinman, 1998; Pulendran et al., 2001). By actively controlling this microenvironment it may be possible to drive the immune response, as demonstrated by Singh et al., using short interfering RNA (siRNA) to silence the expression of IL-10 in order to generate a T_H1 type immune response (Park et al., 2013; Sato et al., 1999; Singh et al., 2008). In this way, by silencing expression of IL-10 on the dendritic cell level, Singh was able to show that this was enough to drive the type of immune response desired without having to manipulate multiple cell populations. By combining new recent findings in immunology along with the basic concept of a protein based vaccine, it may be possible to preferentially drive the immune system to create a desired response.

2.2 Protein Vaccines and Use of Adjuvants

In principle, the simplest vaccine capable of targeting the adaptive immune system would consist of a short peptide encompassing MHC class I-restricted epitopes. However, the use of soluble peptides as vaccines alone has not been successful. While the exact reasons for the poor immunogenicity are not well understood, some studies in mice have demonstrated that, instead of activating T cells, soluble peptides tolerize and/or delete antigen specific T cells (Aichele et al., 1995; Enk et al., 1997; Lutz and Schuler, 2002). A number of strategies have been developed to overcome the weakness of peptides in creating an immune response, specifically usage of adjuvants, lipopeptide conjugation, and direct delivery of peptides to DCs have become popular approaches in developing an immune response (Purcell et al., 2007). Additionally, groups have begun actually modifying the protein peptide to aid in their immunogenicity. One strategy involves chemically modifying the peptide to improve the way that it binds to the MHC

(Cole et al., 2010). Another strategy of this kind involves increasing the strength of the interaction between the epitope and the T cell receptor (Sette and Fikes, 2003). These strategies are not, however, without their complications. Data suggests that while the avidity of the peptide is stronger, using these altered peptides can actually affect the T cell specificity to the intended target cells and, ultimately, each chemically modified epitope needs to be more carefully evaluated to ensure its' specificity to the proteins in question (Cole et al., 2010).

The usage of adjuvants has, therefore, become common. Advances in basic immunology knowledge about how innate immune signals can shape adaptive responses along with improvements in biochemical techniques have led to the design of better adjuvants. Alum, the most commonly used vaccine adjuvant has been in use for the past 80 years (Kool et al., 2011). It is made up of aluminium potassium sulfate and has been shown to induce stronger antibody production. Currently is can be found in several vaccines on the market including: DTP (Diptheria-Tetanus-Pertussis combination), Pediarix (DTP-HBV-Polio combination), Pentacel (DTP-*Haemophilus influenza* B (HIB)-Polio combination), Hepatitis A, HBV, HPV, HIB, and pneumococcal vaccines (Kool et al., 2011). Pediarix currently in use in the hepatitis B vaccine, which is comprised of the recombinant surface antigen for hepatitis B (HBsAg) adsorbed to alum, the adjuvant. However, safety concerns for alum and other adjuvants have been expressed due to reports of side effects, such as nodule formation, local toxicity and inflammation (Trollfors et al., 2005). Additionally, alum based adjuvants induce only a T_H2 specific response and is therefore ultimately limited in its applications. Of note, the exact mechanisms that result in the adjuvantcy of alum remains largely elusive (as reviewed in (Kool et al., 2011)) and discovering the exact mechanism of action that alum has on DCs may help to improve overall adjuvanticity of alum.

MF59 is a squalene-based oil-in-water emulsion formulation that also has been licensed for human use. In trials for a bird flu vaccine, MF59 was shown to induce antibody responses comparable to levels indicative of protection whereas soluble antigen failed to elicit any response (Nicholson et al., 2001). The only negative side effects reported were complaints of pain at injection sights where most were of short duration (Nicholson et al., 2001). Mechanistically, it has been shown that MF59 is a more potent activator of immune related genes than alum and CpG (Mosca et al., 2008). This suggests that in addition to delivering the antigen that MF59 also induces a strong immune-stimulating response directly at the site of injection by inducing the production of immune mediators and triggering the recruitment of CD11b+ monocytes (Mosca et al., 2008) in this way acting as a multi-functional vaccine adjuvant.

Incomplete Freund's Adjuvant, another well researched adjuvant, was changed in 2006 as a result of concerns about prion contamination, changing the formula from beef-derived to an olive-derived one While the olive-derived IFA is better tolerated in human trials (the beef derived IFA actually was shown to cause severe local skin reactions) it also failed to produce the same immunization seen in the beef derived IFA (Rosenberg et al., 2010).

One possible way to cause an immune response is to use our existing knowledge of TLR agonists to exploit the signaling pathways that DCs use to design a synthetic ligand that can target the TLRs more safely than a pathogen-derived ligand. One popular agonist researched is CpG (Davis et al., 1998; Hartmann et al., 1999; Klinman et al., 1999; Krieg, 2002). Unmethylated CpG DNA is a known TLR9 agonist because most of the mammalian geneome is methylated but bacteria lack CpG methylation enzymes, thereby creating a PAMP. CpG, as used in the terms of an adjuvant, refers to unemethylated cytidine-guanosine dinucleotides within a specific pattern of flanking

bases. Dendritic cells naturally recognize this material and associate it with being in the presence of bacterial DNA. CpG has been shown to induce stronger systemic responses (i.e. more widespread responses as compared to MF59's responses which tend to be more local) compared with MF59 and alum (Mosca et al., 2008). Of note, there exists a species-specific difference in the optimal CpG motif for activation (Lipford et al., 1997). There exists many more TLRs both on the surface of dendritic cells as well as on their endosomes (**Table 2.2**). Their location correlates with the type of molecule that it acts to recognize. These may present further potential for future adjuvant design. Further, the delivery system used for protein/adjuvant delivery itself can act as a potent immune adjuvant especially in particulate based systems.

2.3 Charged Particles For Drug Delivery

The charge of a particle can affect the way that the particles are recognized and taken up by cells. The surface of a cell is anionic, so any type of charge that a particle or material has can affect the way the material interacts with the surface of a cell. For example, using a model particle system (polystyrene spheres) cationically modified particles demonstrated a nearly ten-fold increase in cellular interaction as compared to non-modified anionic counterparts, most likely due to electrostatic interactions of the positively charged particles with the negatively charged cell membrane (Foged et al., 2005). The increase in the particle-cell interaction may ultimately lead to an increase in non-specific cellular internalization of particles by DCs because they internalize exogenous antigens by fluid-phase pinocytosis in addition to receptor-mediated endocytosis. Similarly, when PLGA nanoparticles were functionalized with protamine (with an encapsulated model antigen, OVA) it was shown that the cationic

functionalization increased not only the uptake of the particles by BMDCs but also resulted in a significant increase in BMDC surface activation markers as well as secretion of cytokines indicative of a Th1 type immune response (Han et al., 2011). Interestingly, when investigating cationic polymers (polyethylenimine (PEI), polylysine, cationic dextran and cationic gelatin) the polymers alone were shown to cause in increase in cytokine production associated with Th1 type response by activating the TLR-4 pathway (Chen et al., 2010). Interestingly, when looking at many of the same cationic materials (aminodextran, chitosan, PEI, poly(L-lysine) and protamine) coated onto PLGA microparticles showed that the particles alone did not induce any significant amount of differentiation in DCs and, further, did not alter the way DCs differentiated when in the presence of stimulatory molecules (Fischer et al., 2007).

Cationic microparticles have been extensively researched for their usage in DNA vaccine systems and have been shown to be effective delivery systems for surface adsorbed-DNA based vaccines capable of inducing significantly enhanced antibody and CTL responses to the plasmid (Singh et al., 2000). Specifically, the antigen encoded in the plasmid was presented as a peptide epitope on the MHC class I molecule and, further, was shown to be more a more efficient transfection reagent then lipofectamine (a commercially available non-viral transfection reagent) (Denis-Mize et al., 2000). The effects seen were thought to have been a result of endosomal disruption and increased cytoplasmic or nuclear localization (Denis-Mize et al., 2000). The adjuvant effects of CpG (while delivered with p55 protein antigen on a separate particle) were seen only when it was administered in combination (surface loaded) with cationic PLGA particles (Singh et al., 2001)). This increase in activation observed when CpG was surface loaded on a particle as compared to soluble is in agreement with other studies investigating

different particulate based systems and with different antigens (Cai et al., 2008) (Zwiorek et al., 2008).

Polyethyleneimine (PEI) has been recognized among non-viral cationic transfection reagents as one of the most efficient vectors for delivering DNA to the cell nucleus while avoiding lysosomal degradation, a key limiting factor in cellular transfection (Godbey et al., 1999). Linear PEI has been shown, however, to be cytotoxic when administered at high enough dosages to result in successful cellular transfection (Chollet et al., 2002). Adsorbed PEI onto PLA microparticles is capable of transfecting cells *in vitro* as well as offering protection of surface loaded DNA from DNAse degradation (Basarkar et al., 2007). Further, PEI coated PLGA nanoparticles were shown to be able to efficiently deliver surface loaded siRNA to cells *in vitro* and silence the production of proteins in a cell line (Wang et al., 2010). Interestingly, when PLGA nanoparticles were prepared using a single step-PEI coating procedure (i.e. the particles were coated with PEI during initial nanoparticle formation as compared to coating post-nanoparticle formation) they showed higher transfection capability and showed an increase in cell viability as compared to non-adsorbed PEI (Shau et al., 2012). Simply adsorbing cationic molecules to the surface of PLGA microparticles can sometimes, however, be unstable, with the cationic material desorbing (Wischke et al., 2006). It is with this in mind that our laboratory focused on actually conjugating a cationic polymer to the surface of our particles. Our lab has found that by conjugating PEI to the surface of PLGA microparticles we are able to greatly reduce the cytotoxicity usually associated with PEI (Kasturi et al., 2005).

Anionic particles for the delivery of cationic proteins is, though not as widely studied, an emerging field. A novel nanoparticle system made from a combination of PLGA and poly(styrene-co-4-syrene-sulfonate) (PSS) was used to load cationic model

antigens (lysozyme and BSA) showed that negatively charged particles were capable of loading the protein and showed that its ability to load was directly increased as a function of the anionic polymer (PSS). Further they systematically altered protein loading conditions (pH, ionic concentrations) and were able to maximize loading efficiency using these two buffer conditions by driving electrostatic interactions (Cai et al., 2008).

2.4 SURFACE LOADING ON PARTICLES

Biodegradable polymeric particles have received considerable attention as a possible means for delivering vaccine antigens. PLGA has been extensively investigated as a possible vaccine delivery agent because it is biocompatible and resorbablity. In fact, PLGA has already been commercialized for the delivery of protein and peptide-based drugs. Further, PLGA has been widely used to deliver encapsulated protein antigens where by delivering a time-released protein may offer several advantages including eliminating the need for booster vaccinations (Jiang et al., 2005). A major problem hindering the progression of microparticle-based vaccine formulations for human use is the issue of antigen stability during microencapsulation, storage, and release (as reviewed (Weert et al., 2000; Ye et al., 2010)). In the case of a protein-based vaccine, preserving the protein structure is one of the primary concerns and is an impediment in microencapsulation processes currently used. Further, it has been suggested, as in the case of PLGA particles, a local pH drop inside the microparticles due to trapped acid which is part of the PLGA degradation products can actually further increase protein instability and damage the protein structure (as reviewed in (Weert et al., 2000; Ye et al., 2010)). Because surface adsorption of proteins can be done under milder conditions this technique offers an alternative to the harsh and possibly degradative conditions

associated with microencapsulation (O'Hagan et al., 2004; Singh et al., 2004a, 2004b, 2004c). Although studies have suggested that protein adsorption is primarily controlled by electrostatic interactions, hydrophobic interactions between the protein and particle surface can also affect protein loading (Cui et al., 2004; Soppimath et al., 2001). Protein loading will, however, be limited by the actual surface size of the particle. In spite of these limitations, formulations have performed well in animal models eliciting enhanced humoral and cellular immune responses to model bacterial and viral antigens (Ataman-Önal et al., 2006; Cui et al., 2004; Jung et al., 2001; Kazzaz et al., 2000). More recently it has been shown that two different antigens can be adsorbed together onto particulate carriers. The structural integrity of the proteins was extremely well maintained following adsorption and the particulate formulation was immunogenic for both antigens in vivo (Lamalle-Bernard et al., 2006).

2.5 PLASMA MODIFICATION

Chemical methods can have a variety of potential drawbacks. In the case of particles with encapsulated materials, it may be possible that during a wet chemical reaction, hydrophilic encapsulated molecules may begin to release from the microparticles lowering the overall encapsulation efficiency. Chemical modification of the surface of degradable polymeric particles may also result in the partial degradation and scission of the exposed polymer at the surface, leading to accelerated degradation when the material is actually injected within the body. Plasma can be defined as a partially ionized gas and consists of a collection of ions, free radicals, and electrons produced when a gas is transformed into a high energy, excited state by exposure to an energy source under the right physical conditions. Its applications in biomaterial

engineering have widely increased and have been recently reviewed by Desmet et al. (Desmet et al., 2009).

Plasma treatment of a surface can have a variety of consequences. Typically, the surface wettability is increased, functional groups are introduced, roughness is increased, and chain scission and/or cross-linking can occur. The exact functionalities that may be introduced can be altered by using different gases as the source of plasma during treatment. O2 plasma treatment has been shown to introduce oxygen-containing functional groups, such as carboxylic acid groups, peroxide groups, and hydroxyl groups, into the polymer backbone. On the other hand, free radicals are introduced in the backbone of polymers that are treated with Ar or He plasma. When these free radicals are subjected to the atmosphere peroxides and hydroperoxides will be formed on their surface. The introduced functionalities can be subsequently used to bind other molecules to the surface. In addition to increasing hydrophilicity, plasma treated PLGA films have been shown to adsorb more protein then non-treated films (Lee et al., 2007). Furthermore, assuming conditions are optimized, plasma modification appears to have little effect on the degradation profile of plasma treated PLGA films (Holy et al., 2001). Moreover, when compared to wet chemical modification methods to achieve the same surface functionality, plasma modification has been shown to be less detrimental to 3D structure (Djordjevic et al., 2008). These material properties are greatly affected by the type of plasma treatment, gases used, and duration of plasma exposure. Surface roughness (as characterized by atomic force microscopy) of O2 plasma treated PLGA films, for example, showed significant increases in inter-peak width and valley depth dependent on the plasma exposure time. Furthermore, it was shown that cell attachment to these surfaces was also dependent on plasma treatment time, where samples exposed for the longest period of time (20 minutes) resulted in reduced cell attachment as

compared to cell attachment to surfaces exposed for shorter times (though still showed an increase in cell attachment as compared to non-treated PLGA controls) (Wan et al., 2004). Ultimately, the focus of the delivery system needs to be in end application. Each particulate based system needs to be specifically tailored to the disease model in question, the choice of antigen as well as adjuvant must be specific to the disease state and will ultimately dictate the type of particle that will be most beneficial for delivery.

2.6 CANCER VACCINES

Cancer is currently the second most common cause of death in the United States with 580,350 Americans expected to die of cancer in 2013 (American Cancer Society, 2013). Further, The National Institute of Health estimates that the over-all costs of cancer in 2008 were up to $201.5 billion (American Cancer Society, 2013). While survival rates have increased, obvious actions need to be taken to prevent and treat cancer. Vaccination strategies in cancer immunotherapy research have been focused mostly in developing prophylactic therapies to prevent cancer, therapeutic vaccines to destroy already developed tumors, as well as post-surgery treatment (for treatment after solid tumors have been excised to prevent tumors from re-growing) and focus on developing a T cell driven immune response. T cell based immunity is more attractive than humoral immunity because anti-bodies work by targeting proteins that need to be expressed on the surface of cells whereas T cells can target peptides derived from intracellular proteins. All of these therapies have had their issues but hold promise for further development.

Therapeutic cancer vaccines need to act in the same way that a vaccine would for a microbial pathogen, inducing an immune response against the body's own (and thus

non-immunogenic) cells. As reviewed by Rosenberg *et al* the field of cancer immunotherapy when it comes to vaccines is a booming field, with hundreds of vaccine clinical trials in patients despite the absence of convincing supporting animal data that the vaccines alone could affect invasive, vascularized tumors (Rosenberg et al., 2004). Part of the problems in using vaccines in a therapeutic manner after tumor removal is that the microenvironment before/during initial tumor growth progression and for a recurrent tumor is very different (Predina et al., 2013). These changes might be a result of a number of different events including a shift in the dominating population of immune cells or changes in cytokine production locally as a result of the bodies' own wound healing response (Predina et al., 2013). These results indicate that a treatment based vaccine system for recurrent tumors may need to modulate the local tumor microenvironment differently. Currently, one of the most therapeutically relevant treatments is one that is DC based, Sipuleucel-T.

2.7 Dendritic Cells as Vaccines

Since Ralph Steinman's discovery of DCs in 1973, their importance in immunity has been undeniable and they have emerged as a potential target cell population given their ability to induce both the innate and adaptive immune response despite having a misleadingly low number (1%) of the overall cell population (as reviewed recently by Palucka et al. (Palucka and Banchereau, 2012)). While pathogens found in the human body have found ways to hijack DCs to manipulate the immune system only recently have immunologists begun to attempt to do the same to design vaccines (Pulendran et al., 2001). By studying how microbes are capable of manipulating DC function as well as the receptors and signaling pathways through which they act and then using this gained

knowledge to, in turn, manipulate DCs in a controlled manner it may be possible to move forward in rational vaccine design and eventually manipulate the appropriate DC subpopulations in vivo (Pulendran et al., 2001). DCs pulsed, ex vivo, with antigens have been shown to offer protective immunity that is antigen specific and, further, the immunity offered was dependent on $CD8^+$ T cells (Celluzzi et al., 1996).

Sipuleucel-T is a cellular based vaccine consisting of autologous peripheral-blood mononuclear cells activated with a novel recombinant fusion protein. The fusion protein, called PA2024 consists not only of the antigen (one for prostate cancer) but also prostatic acid phosphatase and both are fused to granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF itself has been widely investigated for usage in cancer vaccines given that it has demonstrated an enhanced protection from tumor re-challenge (where the GM-CSF was produced by transfected tumor cells). Interestingly, as reviewed in Klebanoff et al.'s review of cancer vaccines, most clinical trials involving GM-CSF actually had detrimental effects in the vaccine systems it was tested with instead of having the adjuvant effect expected (Klebanoff et al., 2011). Sipuleucel-T was shown to increase a patients' life by 4.1 months as compared to the placebo group (Kantoff et al., 2010).

Research has also been done on developing a therapeutic dendritic cell based vaccine for HIV treatment (García and Routy, 2011). While vaccinations of this type have been shown to enhance HIV-specific immune responses, there was no clear indication that there was any improved clinical outcome in patients, theorized to be a result of an immune system that was already far too damaged by the disease for the vaccine to make any measurable difference (Allard et al., 2012). That being said, a measurable increase in immune cells specific to the proteins the DCs had been transfected to express indicates that this may be a therapy worth continuing investigation.

In another DC based approach, DCs were treated *ex vivo* with autologous heat-inactivated HIV-1 before being delivered to the patient (García et al., 2013). Using this therapy they showed significant decreases in plasma viral load accompanied by increases in T cells specific for HIV-1 .

Table 2.1: Vaccines Currently Available and Those in Advanced Clinical Trials

Subunits
Influenza
Rabies
Anthrax
Purified Proteins
Diptheria
Tetanus
Hepatitis B
Pertussis acellular
Lyme disease
HIV
Protein-conjugated polysaccharides
Haemophilus influenza type B
Pneumococcus
Meningococcus group C
Streptococcus group B
Vectored antigens
Papilloma virus
HIV

This table was modified from the table (Plotkin, 2003) found in The Vaccine Book and is meant for demonstrative purposes and is not meant to be a comprehensive list of vaccines.

Table 2.2: Potential TLR Targets for Adjuvants

Types	Examples	T_H response	TLR target
Surface TLRs and agonists	Triacyl lipopeptides, Pam3Cys	T_H0, T_H2	TLR 2
	Diacyl lipopeptides, Lipoteichoic acids, Zymosan	T_H0, T_H2	TLR 2
	Peptidoglycan, muropeptides, MDP		TLR 2
	LPS, MPL, LPS analogues, Taxol	T_H1	TLR 4
	Flagellin	T_H1	TLR 5
	Uropathogenic bacteria, *Toxoplasma* profiling	T_H1	TLR 11
Endosome TLRs and agonists	dsRNA, Poly(I:C)	T_H1	TLR 3
	Unmethylated CpG DNA, synthetic oligonucleotides	T_H1	TLR 9
	Imidazoquinoline		TLR 7 TLR 8

This chart was modified from a figure from (Guy, 2007; Leleux and Roy, 2013a).

Table 2.3: Challenges in vaccine biology requiring improved control of antigen presentation

Enhance antibody and T cell-mediated immune memory
Improve the quality of the T cell response
Design therapeutic as well as preventative vaccines
Identify vaccines that dampen immunity

This table was modified from Stienman et. al.'s review (Steinman and Pope, 2002)

Table 2.4: Peptide vaccine immunization of patients with metastatic cancer

Peptide	Total Patients	NR	PR	CR
MART-1_{27-35}	23	22	1	0
MART-2_{27-35}+IL-12	23	12	0	0
$gp100_{209-217}$	9	8	0	1
G209-2M + MART-27L	23	23	0	0
G209-2M, g280-9V, MART-27L[c]+tyr3D[d]	16	14	2	0
Dendritic cells + g209-2M + MART-27L	15	13	2	0

*This list is meant to be demonstrative not exhaustive. Adapted from (Rosenberg et al., 2004). Abbreviations are as follows: CR are patients demonstrating complete responsiveness, PR are patients demonstrating partial responsiveness, and NR are patients demonstrating no response.

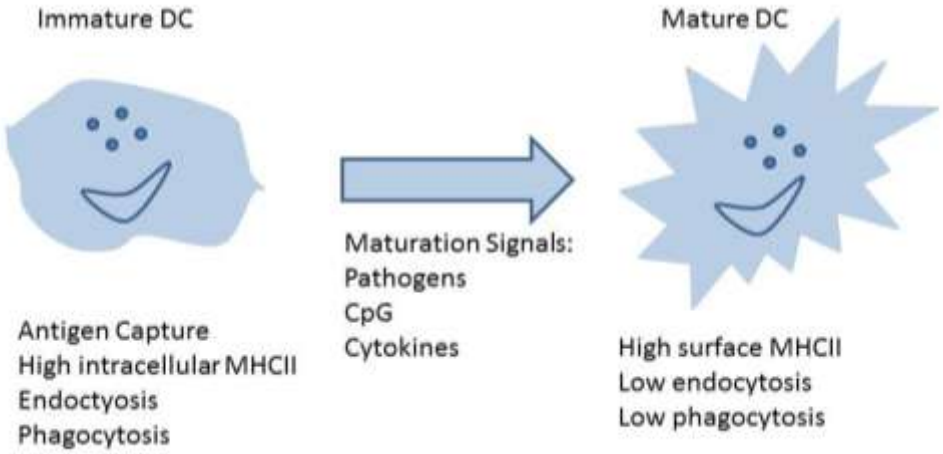

Figure 2.1 Dendritic Cell maturation: Modified from (Banchereau et al., 2000) to represent main differences in function of immature and mature dendritic cells and the maturation signals necessary to drive that differentiation.

2.8 REFERENCES

Aichele, P., Brduscha-Riem, K., Zinkernagel, R.M., Hengartner, H., and Pircher, H. (1995). T cell priming versus T cell tolerance induced by synthetic peptides. J Exp Med 182, 261–266.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nat Rev Immunol 4, 499–511.

Allard, S.D., De Keersmaecker, B., de Goede, A.L., Verschuren, E.J., Koetsveld, J., Reedijk, M.L., Wylock, C., De Bel, A.V., Vandeloo, J., Pistoor, F., et al. (2012). A phase I/IIa immunotherapy trial of HIV-1-infected patients with Tat, Rev and Nef expressing dendritic cells followed by treatment interruption. Clinical Immunology 142, 252–268.

American Cancer Society (2013). Cancer Facts & Figures 2013.

Arnon, R., and Horwitz, R.J. (1992). Synthetic peptides as vaccines. Current Opinion in Immunology 4, 449–453.

Ataman-Önal, Y., Ataman-&Ouml, nal, Y., Munier, S., verine, Gan&eacute, e, A., Terrat, C., line, Durand, P.-Y., et al. (2006). Surfactant-free anionic PLA nanoparticles coated with HIV-1 p24 protein induced enhanced cellular and humoral immune responses in various animal models. Journal of Controlled Release 112, 175–185.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature 392, 245–252.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.-J., Pulendran, B., and Palucka, K. (2000). Immunobiology of Dendritic Cells. Annual Review of Immunology 18, 767–811.

Basarkar, A., Devineni, D., Palaniappan, R., and Singh, J. (2007). Preparation, characterization, cytotoxicity and transfection efficiency of poly(dl-lactide-co-glycolide) and poly(dl-lactic acid) cationic nanoparticles for controlled delivery of plasmid DNA. International Journal of Pharmaceutics 343, 247–254.

Bharali, D.J., Pradhan, V., Elkin, G., Qi, W., Hutson, A., Mousa, S.A., and Thanavala, Y. (2008). Novel nanoparticles for the delivery of recombinant hepatitis B vaccine. Nanomedicine: Nanotechnology, Biology and Medicine 4, 311–317.

Van der Bruggen, P., Stroobant, V., Vigneron, N., and Van den Eynde, B. (2013). Peptide database: T cell-defined tumor antigens.

Cai, C., Bakowsky, U., Rytting, E., Schaper, A.K., and Kissel, T. (2008). Charged nanoparticles as protein delivery systems: A feasibility study using lysozyme as model protein. European Journal of Pharmaceutics and Biopharmaceutics 69, 31–42.

Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature 388, 782–787.

Celluzzi, C.M., Mayordomo, J.I., Storkus, W.J., Lotze, M.T., and Falo, L.D. (1996). Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. J Exp Med 183, 283–287.

Chen, H., Li, P., Yin, Y., Cai, X., Huang, Z., Chen, J., Dong, L., and Zhang, J. (2010). The promotion of type 1 T helper cell responses to cationic polymers in vivo via toll-like receptor-4 mediated IL-12 secretion. Biomaterials 31, 8172–8180.

Chollet, P., Favrot, M.C., Hurbin, A., and Coll, J.-L. (2002). Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. J Gene Med 4, 84–91.

Cole, D.K., Edwards, E.S.J., Wynn, K.K., Clement, M., Miles, J.J., Ladell, K., Ekeruche, J., Gostick, E., Adams, K.J., Skowera, A., et al. (2010). Modification of MHC Anchor Residues Generates Heteroclitic Peptides That Alter TCR Binding and T Cell Recognition. J Immunol 185, 2600–2610.

Cui, Z., Patel, J., Tuzova, M., Ray, P., Phillips, R., Woodward, J.G., Nath, A., and Mumper, R.J. (2004). Strong T cell type-1 immune responses to HIV-1 Tat (1–72) protein-coated nanoparticles. Vaccine 22, 2631–2640.

Davis, H.L., Weeranta, R., Waldschmidt, T.J., Tygrett, L., Schorr, J., and Krieg, A.M. (1998). CpG DNA Is a Potent Enhancer of Specific Immunity in Mice Immunized with Recombinant Hepatitis B Surface Antigen. J Immunol 160, 870–876.

Denis-Mize, K.S., Dupuis, M., MacKichan, M.L., Singh, M., Doe, B., O'Hagan, D., Ulmer, J.B., Donnelly, J.J., McDonald, D.M., and Ott, G. (2000). Plasmid DNA adsorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells. Gene Therapy 7, 2105–2112.

Desmet, T., Morent, R., De Geyter, N., Leys, C., Schacht, E., and Dubruel, P. (2009). Nonthermal plasma technology as a versatile strategy for polymeric biomaterials surface modification: a review. Biomacromolecules 10, 2351–2378.

Djordjevic, I., Britcher, L.G., and Kumar, S. (2008). Morphological and surface compositional changes in poly(lactide-co-glycolide) tissue engineering scaffolds upon radio frequency glow discharge plasma treatment. Applied Surface Science 254, 1929–1935.

Fischer, S., Uetz-von Allmen, E., Waeckerle-Men, Y., Groettrup, M., Merkle, H.P., and Gander, B. (2007). The preservation of phenotype and functionality of dendritic cells upon phagocytosis of polyelectrolyte-coated PLGA microparticles. Biomaterials 28, 994–1004.

Foged, C., Brodin, B., Frokjaer, S., and Sundblad, A. (2005). Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model. Int J Pharm 298, 315–322.

García, F., and Routy, J.-P. (2011). Challenges in dendritic cells-based therapeutic vaccination in HIV-1 infection: Workshop in dendritic cell-based vaccine clinical trials in HIV-1. Vaccine 29, 6454–6463.

García, F., Climent, N., Guardo, A.C., Gil, C., León, A., Autran, B., Lifson, J.D., Martínez-Picado, J., Dalmau, J., Clotet, B., et al. (2013). A Dendritic Cell–Based Vaccine Elicits T Cell Responses Associated with Control of HIV-1 Replication. Sci Transl Med 5, 166ra2–166ra2.

Godbey, W.T., Wu, K.K., and Mikos, A.G. (1999). Poly(ethylenimine) and its role in gene delivery. Journal of Controlled Release 60, 149–160.

Guy, B. (2007). The perfect mix: recent progress in adjuvant research. Nat Rev Micro 5, 505–517.

Han, R., Zhu, J., Yang, X., and Xu, H. (2011). Surface modification of poly(D,L-lactic-co-glycolic acid) nanoparticles with protamine enhanced cross-presentation of encapsulated ovalbumin by bone marrow-derived dendritic cells. Journal of Biomedical Materials Research Part A 96A, 142–149.

Hartmann, G., Weiner, G.J., and Krieg, A.M. (1999). CpG DNA: A potent signal for growth, activation, and maturation of human dendritic cells. PNAS 96, 9305–9310.

Holy, C.E., Cheng, C., Davies, J.E., and Shoichet, M.S. (2001). Optimizing the sterilization of PLGA scaffolds for use in tissue engineering. Biomaterials 22, 25–31.

Hubbell, J.A., Thomas, S.N., and Swartz, M.A. (2009). Materials engineering for immunomodulation. Nature 462, 449–460.

Jiang, W., Gupta, R.K., Deshpande, M.C., and Schwendeman, S.P. (2005). Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. Advanced Drug Delivery Reviews 57, 391–410.

Jung, T., Kamm, W., Breitenbach, A., Hungerer, K.-D., Hundt, E., and Kissel, T. (2001). Tetanus Toxoid Loaded Nanoparticles from Sulfobutylated Poly(Vinyl Alcohol)-Graft-Poly(Lactide-co-Glycolide): Evaluation of Antibody Response After Oral and Nasal Application in Mice. Pharm Res 18, 352–360.

Kantoff, P.W., Higano, C.S., Shore, N.D., Berger, E.R., Small, E.J., Penson, D.F., Redfern, C.H., Ferrari, A.C., Dreicer, R., Sims, R.B., et al. (2010). Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer. New England Journal of Medicine 363, 411–422.

Kasturi, S.P., Sachaphibulkij, K., and Roy, K. (2005). Covalent conjugation of polyethyleneimine on biodegradable microparticles for delivery of plasmid DNA vaccines. Biomaterials 26, 6375–6385.

Kazzaz, J., Neidleman, J., Singh, M., Ott, G., and O'Hagan, D.T. (2000). Novel anionic microparticles are a potent adjuvant for the induction of cytotoxic T lymphocytes against recombinant p55 gag from HIV-1. J Control Release 67, 347–356.

Klebanoff, C.A., Acquavella, N., Yu, Z., and Restifo, N.P. (2011). Therapeutic cancer vaccines: are we there yet? Immunological Reviews 239, 27–44.

Klinman, D.M., Barnhart, K.M., and Conover, J. (1999). CpG motifs as immune adjuvants. Vaccine 17, 19–25.

Kool, M., Fierens, K., and Lambrecht, B.N. (2011). Alum adjuvant: some of the tricks of the oldest adjuvant. Journal of Medical Microbiology 61, 927–934.

Krieg, A.M. (2002). Cpg Motifs in Bacterial Dna and Their Immune Effects*. Annual Review of Immunology 20, 709–760.

Lamalle-Bernard, D., Munier, S., Compagnon, C., Charles, M.-H., Kalyanaraman, V.S., Delair, T., Verrier, B., and Ataman-Önal, Y. (2006). Coadsorption of HIV-1 p24 and gp120 proteins to surfactant-free anionic PLA nanoparticles preserves antigenicity and immunogenicity. Journal of Controlled Release 115, 57–67.

Lee, S.G., An, E. young, Lee, J.B., Park, J.C., Shin, J.W., and Kim, J.K. (2007). Enhanced cell affinity of poly(d,l-lactic-co-glycolic acid) (50/50) by plasma treatment with β-(1 → 3) (1 → 6)-glucan. Surface and Coatings Technology 201, 5128–5131.

Leleux, J., and Roy, K. (2013). Micro and Nanoparticle-Based Delivery Systems for Vaccine Immunotherapy: An Immunological and Materials Perspective. Advanced Healthcare Materials 2, 72–94.

Lipford, G.B., Bauer, M., Blank, C., Reiter, R., Wagner, H., and Heeg, K. (1997). CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. Eur. J. Immunol. 27, 2340–2344.

Mahnke, K., Schmitt, E., Bonifaz, L., Enk, A.H., and Jonuleit, H. (2002). Immature, but not inactive: the tolerogenic function of immature dendritic cells. Immunol Cell Biol 80, 477–483.

Mosca, F., Tritto, E., Muzzi, A., Monaci, E., Bagnoli, F., Iavarone, C., O'Hagan, D., Rappuoli, R., and Gregorio, E.D. (2008). Molecular and cellular signatures of human vaccine adjuvants. PNAS 105, 10501–10506.

Nicholson, K.G., Colegate, A.E., Podda, A., Stephenson, I., Wood, J., Ypma, E., and Zambon, M.C. (2001). Safety and antigenicity of non-adjuvanted and MF59-

adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. The Lancet 357, 1937–1943.

O'Hagan, D.T., Singh, M., and Ulmer, J.B. (2004). Microparticles for the delivery of DNA vaccines. Immunol. Rev. 199, 191–200.

Palucka, K., and Banchereau, J. (2012). Cancer immunotherapy via dendritic cells. Nat Rev Cancer 12, 265–277.

Park, J., Wu, C.T., and Bryers, J.D. (2013). Chemokine programming dendritic cell antigen response: part I - select chemokine programming of antigen uptake even after maturation. Immunology 139, 72–87.

Pierre, P., Turley, S., Gatti, E., Hull, M., Meltzer, J., Mizra, A., Inaba, K., Steinman, R., and Mellman, I. (1997). Developmental regulation of MHC class II transport in mouse dendritic cells. Nature 388, 787–792.

Plotkin, S. (2003). Disease States and Vaccines: Selected Cases. In The Vaccine Book, (San Diego, California: Academic Press), pp. 179–188.

Predina, J., Eruslanov, E., Judy, B., Kapoor, V., Cheng, G., Wang, L.-C., Sun, J., Moon, E.K., Fridlender, Z.G., Albelda, S., et al. (2013). Changes in the local tumor microenvironment in recurrent cancers may explain the failure of vaccines after surgery. PNAS 110, E415–E424.

Pulendran, B., Palucka, K., and Banchereau, J. (2001). Sensing Pathogens and Tuning Immune Responses. Science 293, 253–256.

Purcell, A.W., McCluskey, J., and Rossjohn, J. (2007). More than one reason to rethink the use of peptides in vaccine design. Nat Rev Drug Discov 6, 404–414.

Rosenberg, S.A., Yang, J.C., and Restifo, N.P. (2004). Cancer immunotherapy: moving beyond current vaccines. Nat Med 10, 909–915.

Rosenberg, S.A., Yang, J.C., Kammula, U.S., Hughes, M.S., Restifo, N.P., Schwarz, S.L., Morton, K.E., Laurencot, C.M., and Sherry, R.M. (2010). Different Adjuvanticity of Incomplete Freund's Adjuvant Derived From Beef or Vegetable Components in Melanoma Patients Immunized With a Peptide Vaccine. Journal of Immunotherapy 33, 626–629.

Rutella, S., Danese, S., and Leone, G. (2006). Tolerogenic dendritic cells: cytokine modulation comes of age. Blood 108, 1435–1440.

Sato, M., Iwakabe, K., Kimura, S., and Nishimura, T. (1999). Functional skewing of bone marrow-derived dendritic cells by Th1- or Th2-inducing cytokines. Immunol. Lett. 67, 63–68.

Sette, A., and Fikes, J. (2003). Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. Current Opinion in Immunology 15, 461–470.

Shau, M.D., Shih, M.F., Lin, C.C., Chuang, I.C., Hung, W.C., Hennink, W.E., and Cherng, J.Y. (2012). A one-step process in preparation of cationic nanoparticles with poly(lactide-co-glycolide)-containing polyethylenimine gives efficient gene delivery. European Journal of Pharmaceutical Sciences 46, 522–529.

Singh, A., Nie, H., Ghosn, B., Qin, H., Kwak, L.W., and Roy, K. (2008). Efficient Modulation of T-cell Response by Dual-mode, Single-carrier Delivery of Cytokine-targeted siRNA and DNA Vaccine to Antigen-presenting Cells. Molecular Therapy 16, 2011–2021.

Singh, M., Briones, M., Ott, G., and O'Hagan, D. (2000). Cationic microparticles: A potent delivery system for DNA vaccines. PNAS 97, 811–816.

Singh, M., Ott, G., Kazzaz, J., Ugozzoli, M., Briones, M., Donnelly, J., and O'Hagan, D.T. (2001). Cationic Microparticles Are an Effective Delivery System for Immune Stimulatory CpG DNA. Pharm Res 18, 1476–1479.

Singh, M., Kazzaz, J., Chesko, J., Soenawan, E., Ugozzoli, M., Giuliani, M., Pizza, M., Rappouli, R., and O'Hagan, D.T. (2004a). Anionic microparticles are a potent delivery system for recombinant antigens from Neisseria meningitidis serotype B. Journal of Pharmaceutical Sciences 93, 273–282.

Singh, M., Chesko, J., Kazzaz, J., Ugozzoli, M., Kan, E., Srivastava, I., and O'Hagan, D.T. (2004b). Adsorption of a novel recombinant glycoprotein from HIV (Env gp120dV2 SF162) to anionic PLG microparticles retains the structural integrity of the protein, whereas encapsulation in PLG microparticles does not. Pharm. Res. 21, 2148–2152.

Singh, M., Kazzaz, J., Ugozzoli, M., Chesko, J., and O'Hagan, D.T. (2004c). Charged polylactide co-glycolide microparticles as antigen delivery systems. Expert Opin Biol Ther 4, 483–491.

Soppimath, K.S., Aminabhavi, T.M., Kulkarni, A.R., and Rudzinski, W.E. (2001). Biodegradable polymeric nanoparticles as drug delivery devices. Journal of Controlled Release 70, 1–20.

Steinman, R.M., and Pope, M. (2002). Exploiting dendritic cells to improve vaccine efficacy. Journal of Clinical Investigation 109, 1519–1526.

Trollfors, B., Bergfors, E., and Inerot, A. (2005). Vaccine related itching nodules and hypersensitivity to aluminium. Vaccine 23, 975–976.

Wan, Y., Qu, X., Lu, J., Zhu, C., Wan, L., Yang, J., Bei, J., and Wang, S. (2004). Characterization of surface property of poly(lactide-co-glycolide) after oxygen plasma treatment. Biomaterials 25, 4777–4783.

Wang, J., Feng, S.-S., Wang, S., and Chen, Z. (2010). Evaluation of cationic nanoparticles of biodegradable copolymers as siRNA delivery system for hepatitis B treatment. International Journal of Pharmaceutics 400, 194–200.

Weert, M. van de, Hennink, W.E., and Jiskoot, W. (2000). Protein Instability in Poly(Lactic-co-Glycolic Acid) Microparticles. Pharm Res 17, 1159–1167.

Wischke, C., Borchert, H.-H., Zimmermann, J., Siebenbrodt, I., and Lorenzen, D.R. (2006). Stable cationic microparticles for enhanced model antigen delivery to dendritic cells. J Control Release 114, 359–368.

Ye, M., Kim, S., and Park, K. (2010). Issues in long-term protein delivery using biodegradable microparticles. Journal of Controlled Release 146, 241–260.

Ben-Yedidia, T., and Arnon, R. (1997). Design of peptide and polypeptide vaccines. Current Opinion in Biotechnology 8, 442–448.

Zwiorek, K., Bourquin, C., Battiany, J., Winter, G., Endres, S., Hartmann, G., and Coester, C. (2008). Delivery by Cationic Gelatin Nanoparticles Strongly Increases the Immunostimulatory Effects of CpG Oligonucleotides. Pharm Res 25, 551–562.

CHAPTER 3

Plasma Surface Modification of PLGA Microparticles for Efficient Anionic Protein Loading

3.1 INTRODUCTION:

This chapter describes work done in collaboration with Dr. Laxminarayana Raja's lab in the Department of Aerospace engineering at the University of Texas at Austin. Briefly, we examined the effects of using plasma technology to alter the surface of PLGA microparticles. Dr. Raja's dielectric barrier glow discharge system was used to create an atmospheric glow discharge plasma (APG). We investigated altering a variety of conditions associated with the formation of this kind of plasma to find conditions that imparted significant changes in zeta potential. We also studied the effects of using a low-pressure plasma system on zeta potential, protein loading, and surface modification over time. These studies offer important and interesting proof-of-concept in using plasma as a dry-chemical process to add functional groups to the surface of microparticles. We showed that the atmospheric system that Dr. Raja developed was able to impart a significant amount of anionic charge on the surface of our PLGA microparticles. This charge varied depending on the frequency of the applied voltage. While the dielectric material is very important in plasma formation, no significant change in particle zeta potential was observed as a function of this variable. Ovalbumin was loaded onto the microparticles at a loading efficiency of 36%. Low pressure plasma was also investigated as an alternative to the APG system for usage. We looked at using different gasses, specifically He and O_2 plasma and while little difference in charge was observed, SEM images revealed a slightly rougher surface on the O_2 plasma exposed particles. Lysozyme was electrostatically attached to the particles at close to 100% loading efficiency. Unfortunately, it seems that the surface of our particles "age" and zeta

potential is lost over time. Thus, more work needs to be done to investigate possible storage solutions for usage of these materials.

3.2 Background and Motivation

The surface properties of materials play a very important role in the overall function and biocompatibility of a material. One major concern when dealing with a material that comes into contact with extracellular fluids is the way that material will interact with proteins found in plasma. Because of this, ultimately you are dealing with a two very separate issues in selecting a single material. The material needs to have specific bulk properties while also interacting favorably with extracellular fluids. This is a difficult set of properties for a single material to meet. For this reason, many different methods of altering surface properties have been investigated, including: coating/adsorption, plasma modification, or chemically grafting another material onto the surface (Ikada, 1994).

Most methods for introducing new functional groups onto the surface of materials are wet chemical based methods. While effective, they can also have many undesirable effects on a material, especially if that material is biodegradable. These modification techniques can result in a loss of mechanical properties and ultimately lead to a faster degradation rate (Desmet et al., 2009; Morent et al., 2011). In this way, a dry plasma based strategy may be beneficial.

Plasma can be thought of as an ionized gas composed of free electrons, ions, and radicals (along with neutral particles). Plasma is formed when gases are excited by applying energy to the gas. It reorganizes the electronic structure of the species and produces excited ions. Plasma reactions themselves are typically broken down into three

categories: plasma polymerization, plasma treatment, and plasma etching (or ablation) (reviewed in (Desmet et al., 2009)). Plasma polymerization involves actually depositing monomers onto the surface of a substrate, and etching occurs when the ions from the plasma strike the substrate hard enough to actually have an ablative reaction (Desmet et al., 2009). Plasmas can be used to insert chemical groups or radicals onto otherwise non-reactive surfaces (as reviewed in (Siow et al., 2006)). Different gasses will introduce different functionalities onto the surface of the materials. He and Ar plasmas typically introduce free radicals to the surface of the substrate, which then react with air and ultimately lead to the formation of peroxides and hydroperoxides. O_2 based plasmas tend to introduce a mixture of carboxylic acid and hydroxide groups (Desmet et al., 2009). Plasma modification has been extensively researched in polymeric films (Desmet et al., 2009; Mattioli et al., 2012; Morent et al., 2011; Wan et al., 2004) but no publications of its usage in particles have been reported.

While most research has focused on using cationic particles for delivering anionic protein antigens and other immunomodulatory molecules, there is need for anionic particles to deliver cationic molecules, especially protein antigens. The first group credited with adsorbing antigens onto the surface of a particle based delivery system was Almeida, Alpar, and Brown (Almeida et al., 1993). PLGA microparticles prepared with anionic surfactants like sodium dodecyl sulfate (SDS) have been shown to be able to adsorb p55 gag protein (from HIV-1) and were able to induce CTL responses in mice as well as antibody responses (Kazzaz et al., 2000). Further, work has been done investigating the usage of anionic nanoparticles to deliver a protein that had been made more positively-charged (the process is referred to as cationization). Interestingly, the cationized protein elicited antibody responses similar to that seen with the non-charged protein delivered with alum but the greatest amount of response was seen when the

cationized protein was delivered adsorbed onto their anionic particles (Cui and Mumper, 2002). Surfactant free PLA nanoparticles surface loaded with the HIV-1 p24 capsid protein were able to induce a CTL response in mice without the need for an additional adjuvant (as compared to the protein delivered in combination with alum) (Ataman-Önal et al., 2006).

Here we propose the usage of PLGA microparticles charge-functionalized using plasma surface modification. We investigated two plasma sources: an atmospheric plasma glow discharge system and a low pressure plasma system. With the help of Laxminarayana Raja's lab we began our investigations using a dielectric barrier discharge system to create atmospheric glow discharge plasma (Shin and Raja, 2003, 2007). This type of system has many advantages from an industrial standpoint, the most compelling of which is its cost. This system eliminates the need for expensive vacuum equipment and can actually be scaled up (depending on the size of the electrodes). The main issue in scale up is that there is a greater chance that instabilities in the plasma will arise, and the plasma may actually create a thermal arc discharge. Again, if this transition occurs the plasma will no longer be homogenous and the plasma can lose its non-thermal nature. This is why the system used creates plasma in a pulsed regimen. Another factor that works to stabilize the plasma in this system is the dielectric material used to cover the electrodes (Tendero et al., 2006). While APG plasmas have a lot of advantages, low pressure systems typically used in semi-conductor work also have their own advantages. Using low pressure removes any contaminating molecules/gases creating a free path for the gas you are using to create the plasma and interact with your material, making your reactions easier to control. For this reason we looked into using a low pressure system. Here we investigated the usage of two different gasses, He and O_2. We investigated a number of factors within the plasma production as well as time of exposure to alter the

zeta potential of our PLGA microparticles. We then used our particle formulations to load model protein antigens on the surface.

3.3 MATERIALS AND METHODS:

3.3.1 Reagents

PLGA Resomer® RG502H, ovalbumin, lysozyme, monosodium phosphate, and disodium phosphate were purchased from Sigma-Aldrich. Poly(vinyl alcohol) PVA MW ~ 31,000 was purchased from Fluka. Micro BCA kit for protein analysis was purchased from (Thermo Scientific, Rockford, IL).

3.3.2 Plasma surface modification using a novel He gas atmospheric plasma glow (APG) discharge system

PLGA microparticles were prepared as we have previously reported (Kasturi et al., 2005; Pai Kasturi et al., 2006; Singh et al., 2008). Briefly, acid end-capped PLGA RG502H (MW ~ 11,000 Da, Sigma-Aldrich) microparticles were prepared using a water-in-oil-in-water (w/o/w) double emulsion, solvent evaporation technique. Briefly, 0.2 g of PLGA was dissolved in 7 mL of dichloromethane (DCM) (Thermo Fischer Scientific Inc., Waltham, MA). 300 μL of deionized water was added to the polymer solution and immediately homogenized for 2 minutes. The emulsion waspoured into a 1% PVA solution and homogenized for 2 minutes and then the DCM was evaporated from the solution. The microparticles were collected and washed with deionized water, lyophilized and stored at -20°C. The microparticles were then coated onto glass slides at a concentration of 2.5 mg/mL (concentration based on slide coating optimization studies, data not shown) in deionized water. Slides were allowed to dry at room temperature until

all water had evaporated (approximately 3 hours) prior to plasma exposure. PLGA microparticles were plasma modified using a novel atmospheric plasma glow (APG) discharge system (Shin and Raja, 2003, 2007). The dielectric barrier discharge system consists of two plane-parallel metal electrodes where the electrodes are covered by a dielectric material (**Figure 3.1**). The two electrodes are separated by a gap that was about 3 cm, and He gas is allowed to flow through that gap at a rate of 4.5 standard liters per minute (SLPM), and the sample is immobilized over one of the electrodes. The discharge was then initiated by a sinusoidally applied power source. An outline of the experimental objectives can be seen in **Figure 3.2**. Because the dielectric layer plays an important role in the plasma process by acting to ensure homogenous plasma formation, and thus a more homogenous surface treatment, we investigated the usage of different dielectric materials in generating anionic surface charge. Zeta potential was then quantified using ZetaPlus (BrookHaven Instruments Corporation, Holtsville, NY). All microparticle samples were suspended in 1 mM KCl.

3.3.3 Low pressure plasma surface treatment of PLGA microparticles

We also investigated a low pressure plasma system for surface modification. Just as in the case of our APG surface modification, we prepared PLGA microparticles as described in section 3.3.1. We coated the microparticles at a concentration of 5 mg/mL on each glass slide. The PLGA microparticle coated slides were then plasma modified at a pressure of 180 mTorr using either oxygen or helium plasma at a rate of 18 SCCM and 50 Watts using the Plasma Therm 790 Series reactive ion etcher. The slides were then stored in a vacuum chamber and microparticles were removed by resuspension in water and either immediately used or lyophilized and stored at -20°C.

3.3.4 Protein loading on plasma treated PLGA microparticles

Ovalbumin or lysozyme (Sigma, Saint Louis, MO) was loaded onto the surface of plasma-modified microparticles at 1.2% (w/w) in 10 mM citrate buffer or 10 mM phosphate buffer. Briefly, plasma modified microparticles were added to a dilute solution of ovalbumin and allowed to incubate at 4°C overnight on an end over end shaker. Adjusting the pH of our loading buffer enabled us to adsorb greater concentrations of protein to our plasma-treated microparticles. Protein concentration was indirectly calculated by measuring the concentration of remaining protein that did not adsorb to the particles using a Micro BCA Protein Assay Kit. In other words, the protein concentration measured in the supernatant remaining after protein adsorption to the particles was subtracted from the initial protein concentration added.

3.3.5 SEM of plasma modified microparticles

Scanning electron microscopy images were taken of the microparticles after low pressure plasma treatment to assess any possible effects (qualitatively) that plasma modification may have had on the surface characteristics of the microparticles. The microparticles were coated onto aluminum stubs obtained from the electron microscopy facility. Microparticles were suspended in 0.2 μm filtered purified water and deposited on the stubs and allowed to dry overnight. The microparticle coated stubs were then coated with 15 nm of gold:palladium using the sputter coater at the core facility. The microparticles were then imaged using the scanning electron microscope (Zeis Supra 40 VP).

3.3.6 Storage conditions

Long term storage options were investigated for our particles. First, we assessed if lyopholization would change the zeta potential. After exposure to plasma (low pressure plasma for 30 seconds using the Plasma Therm as previously described) PLGA microparticles were resuspended in 0.2 μm water and then lyophilized overnight to remove water. Zeta potential analysis was then performed using a Delsa Nano Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Brea, CA). Following lyopholization of particles, particles were stored at -20°C. Zeta potential was then measured at 24 hours and then a week after that to assess if there were any changes in zeta potential. These measurements were done on the Delsa Nano Zeta Petential and Submicron Particle Size Analyzer. All measurements were performed in 1 mM KCl solution.

3.4 RESULTS

3.4.1 Exposing PLGA microparticles to plasma using an APG system can impart a significant change in zeta potential

Our results indicated that no significant difference exists between the zeta potential of particles plasma modified using different dielectric materials (**Figure 3.3**). Previous optimization studies (data not shown) using the APG system suggested that although a variety of parameters could be altered during the plasma modification process, such as gas flow and applied voltage, the only parameter that altered the zeta potential of our microparticles was the frequency with which the voltage was applied (**Figure 3.4**). Therefore frequency of the applied voltage was varied in order to investigate the effects of plasma wave generation on surface charge using poly(methylmethacrylate) as the dielectric material (**Figure 3.4**). We found that treating the PLGA microparticles with

helium atmospheric plasma glow (APG) discharge imparted the microparticles with a significant negative zeta potential ($p<0.01$).

3.4.2 Protein loading onto APG modified PLGA microparticles

As we approached the isoelectric point of ovalbumin (approximately 4.6) we were able to achieve the greatest amount of protein adsorption. The highest concentration of protein adsorption was obtained using citrate buffer at a pH of 5. Under these conditions, approximately 36% loading efficiency was observed; meaning the dosage of OVA would be 4.3 μg/mg of PLGA (**Figure 3.5** and **Figure 3.6**).

3.4.3 Low pressure plasma exposure causes significant change in zeta potential

Low pressure plasma was investigated for usage in functionalizing our particles. Because the PlasmaTherm offered us the ability to easily investigate different gasses, we decided to look at the difference in charge between He plasma modification and O_2 plasma modification. O_2 plasma, aside from introducing different functional groups onto the surface of the particles, has also been shown to be more ablative, which may have an effect on protein loading. While, obviously charge is very important in our studies, other factors are also involved in protein loading and attachment. Interestingly, prolonged exposure of PLGA to plasma actually demonstrated a loss of some polar groups that had been introduced to the surface of the polymer matrix (Wan et al., 2004) which would most likely alter the ability to attach protein to the surface (not to mention the increased surface roughness would also alter protein adsorption). Additionally, because plasma is known to destroy biologically active materials (Laroussi, 2005) we were interested in avoiding prolonged exposure to plasma. Because He plasma gave a lower zeta potential

at a low exposure time (30 seconds) (**Figure 3.8**) we decided to move forward with the remaining protein loading with this exposure time and gas.

3.4.4 Scanning electron microscopy images reveal rougher surfaces on low pressure plasma modified particles

SEM images were taken to provide us with a qualitative prospective of the effects of plasma modification on the roughness of our particles. **Figure 3.9** shows fairly smooth non-modified PLGA microparticles. **Figure 3.10** and **Figure 3.11** both show He modified PLGA microparticles. Here you can see a rougher surface as compared to our non-modified microparticles. Similarly, in O_2 modified PLGA microparticles (**Figure 3.12** and **Figure 3.13**) a rougher surface can be seen. Because this is a more qualitative study, no clear conclusions can be drawn as to whether oxygen or helium plasma is more detrimental to our particles. For that, further studies need to be performed.

3.4.5 Low pressure plasma modified particles can load lysozyme at high efficiency

Lysozyme, a protein that is cationic at physiological pHs (the isoelectric point is 11.3), was used as a model protein to test the binding abilities of our anionic microparticles. We showed that we could bind close to 99% in phosphate buffer at a pH of 7. This is significantly higher then what was observed when we attempted to bind ovalbumin to our particles (the isoelectric point of ovalbumin is about 4.6). This highlights the importance of surface charge on absorption of proteins.

3.4.6 Lyopholization does not alter zeta potential of low pressure modified PLGA microparticles but the zeta potential is unstable

For long term usage of plasma modified particles it must be possible to store them. We investigated if lyopholization changed zeta potential (**Figure 3.7**) and saw no significant change in zeta potential after lyopholization. We then stored the lyophilized particles at -20°C for a week and measured the zeta potential of the same batch of particles. Here we saw significant changes in zeta potential (**Table 3.1**).

3.5 DISCUSSION

Charged particulate delivery of protein antigens via microparticles is a widely researched topic. As reviewed recently by Leleux and Roy (Leleux and Roy, 2013a) particulate carriers are an attractive means for enhancing the delivery of antigens as well as increasing the potency of the vaccines themselves. Here we show a set of studies using plasma to modify the charge of PLGA microparticles in order to offer a new platform to delivery cationic antigenic proteins.

Plasma has been investigated for the usage of biomaterial applications in many different polymer systems (Morent et al., 2011). Here we investigated the usage of two different plasma systems, an APG plasma system and a low pressure plasma system, to modify our PLGA particle systems and, ultimately, load an antigeneic protein on its surface. Plasma modification has been shown to be able to graft proteins as effectively as conventional chemical conjugation methods (Huang et al., 2007). Here, using an appropriately charged protein (**Figure 3.14**), we were able to load close to 99% of the model protein antigen, offering a proof of concept that these particles can load proteins at high efficiency. Since PLGA is a biodegradable polymer, less exposure to solutions (like the ones that would be necessary to perform a wet chemical conjugation) would be most

beneficial in our system. This could prove to be especially beneficial if we were to consider encapsulation within our particle as an additional level of functionality. However, we would need to work on minimizing the exposure of our materials to plasma as it effectively destroys biological molecules (Eisenbrey et al., 2009; Laroussi, 2005).

Because we know that plasma may have ablative/destructive effects on the surface of our particles we qualitatively investigated the effects of the plasma (**Figure 3.9-3.13**). In the case of PLGA films, increasing the exposure time caused a direct increases in the roughness of the surface, and, further, longer treatment times actually resulted in less functional groups (as a result of the surface having been destroyed) (Wan et al., 2004). These results were not exclusive to PLGA based systems (Mattioli et al., 2012). Obvious changes in roughness of our particles were evident in our images, but, again, further analysis needs to be done to make any definitive analysis between differences in level of ablativeness between types of plasma.

Although these results with the APG system were promising, we found that overall the data was more unpredictable then we desired. Specifically, our zeta potential measurements often varied from batch to batch with as much as a 20 mV reduction in anionic charge. Although we had assumed this was due to the gradual "wearing out" of the dielectric material, literature suggests that APG discharge systems similar to that which we are using often produce varied results (Shin and Raja, 2007). This is attributed to instabilities in the discharge itself that ultimately lead to a thermal arc discharge. The transition into a thermal arc discharge can cause a loss of homogeneity of plasma. Additionally, because these systems are effectively open to atmospheric air, other gases can potentially contaminate the plasma. Although an APG system holds promise in an industrial setting in that it can potentially be scaled up to increase the output of PLGA plasma modified particles while eliminating the expensive equipment associated with

more traditional low pressure systems (i.e. cost of vacuum equipment), we decided to investigate the usage of low pressure-based systems for our purposes in order to achieve more consistent results.

Another concern with this system is a well-known plasma-associated phenomenon, plasma aging. Plasma treated surfaces are known to go back to their original state (i.e. the plasma surface modification is not permanent) (Morent et al., 2010; Vesel and Mozetic, 2012). In short, literature indicates that the longer the polymer was treated, the longer the plasma modification lasted. This is most likely associated with an increase in crystallinity at the surface of the material (Vesel and Mozetic, 2012). We have been focusing on shortening the time of exposure to plasma, which may explain the very short duration of the plasma's effects on zeta potential. These studies confirm the results that we observed but were ultimately limited in the conditions tested for storage.

In conclusion we present here a new way of adding anionic surface charge to PLGA microparticles for the delivery of cationic antigeneic proteins. Because this is a dry reaction, it offers the promise of increasing the dosage of any encapsulated agents within the polymeric microparticles.

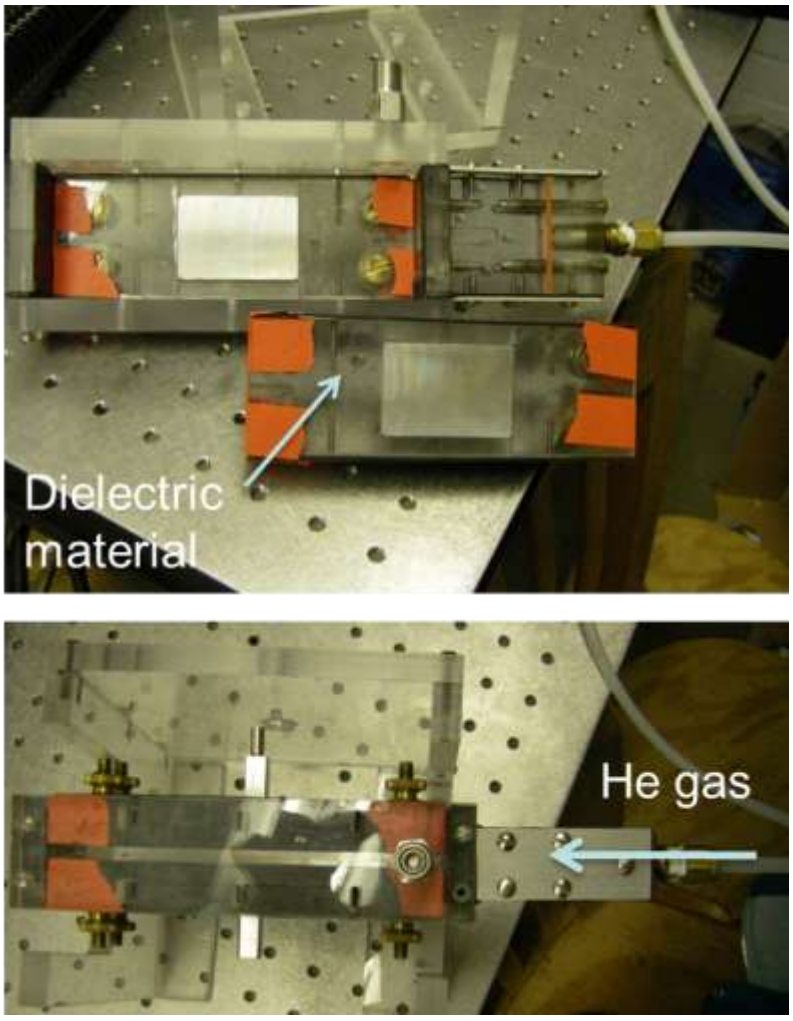

Figure 3.1 Photograph of the atmospheric plasma glow discharge system. The dielectric material covers a parallel plate electrode. Helium gas is flown through the chamber and plasma is generated. This device was loaned to us by Dr. Laxminarayana Raja (Department of Aerospace Engineering, The University of Texas at Austin).

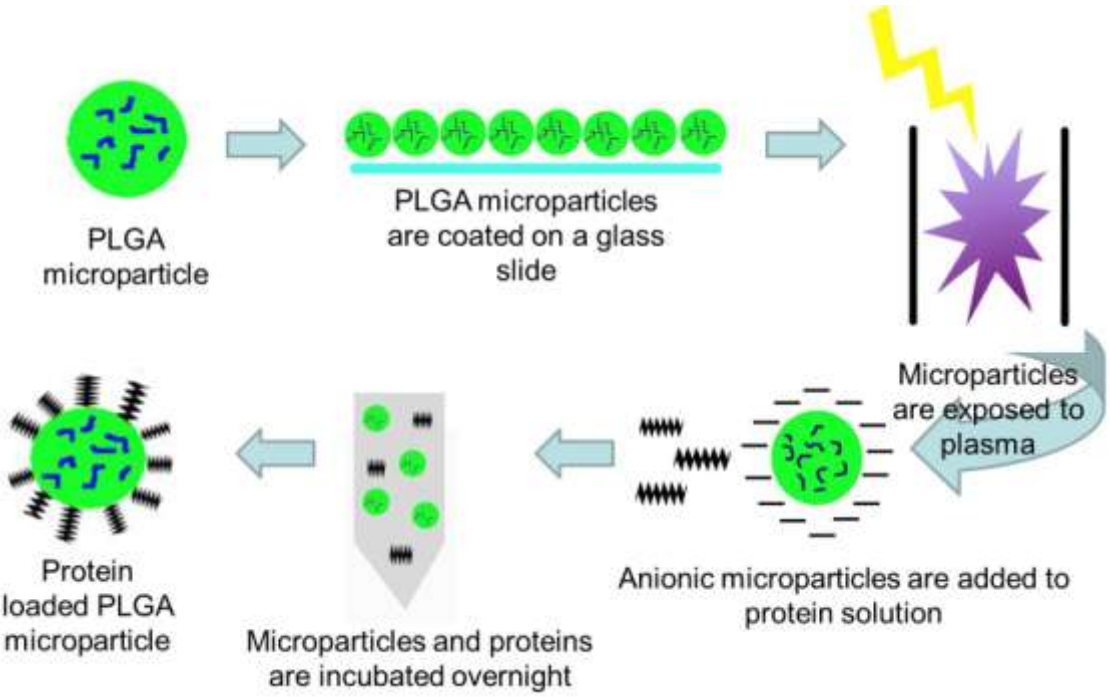

Figure 3.2 An overall schematic of the protein loading experiments for the plasma modified particles. PLGA microparticles are made using a w/o/w emulsion. They are then coated onto glass slides at a concentration of 5 mgs/mL. They are exposed to plasma and harvested from the slides. They are then added to a dilute solution of protein and incubated overnight at 4° C.

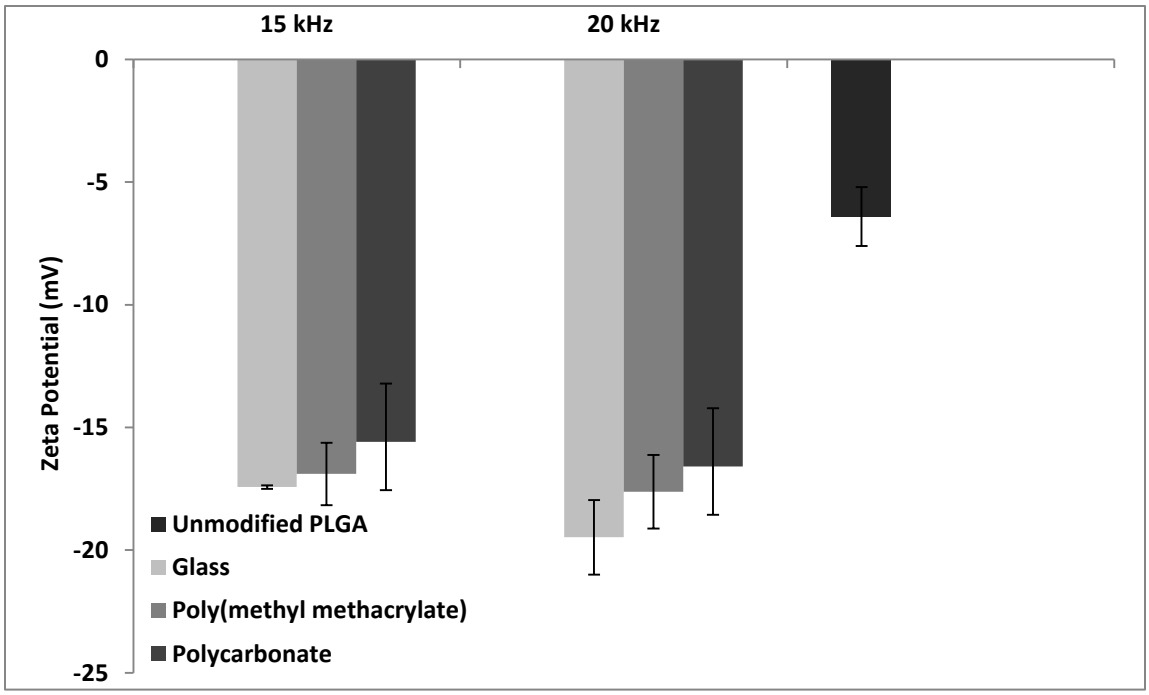

Figure 3.3 Altering dielectric materials does not greatly alter the observed zeta potential of the microparticles. The dielectric layer plays an incredibly important role by acting to stabilize the plasma formed in this type of system specifically by limiting the discharge current and thereby avoiding the arc transition and, further, it acts to distribute any randomly formed streamers on the electrode surface to form a more homogeneous plasma treatment (as reviewed in (Tendero et al., 2006)).

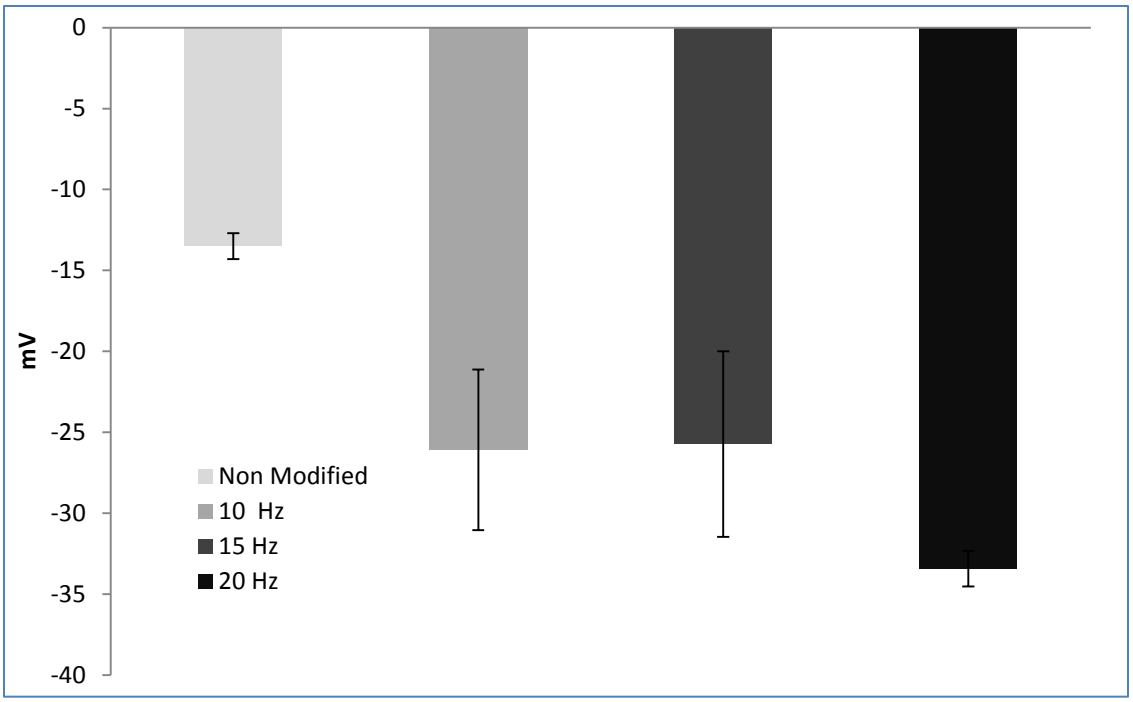

Figure 3.4 Changes in the frequency of the applied voltage alters the zeta potential in the atmospheric pressure glow discharge system. Each slide, coated with 2.5 mgs of PLGA microparticles was exposed to plasma for the same time (6 minutes) with the same gas flow and same voltage of power. The particles were then resuspended in 1 mM KCl and zeta potential analysis was performed.

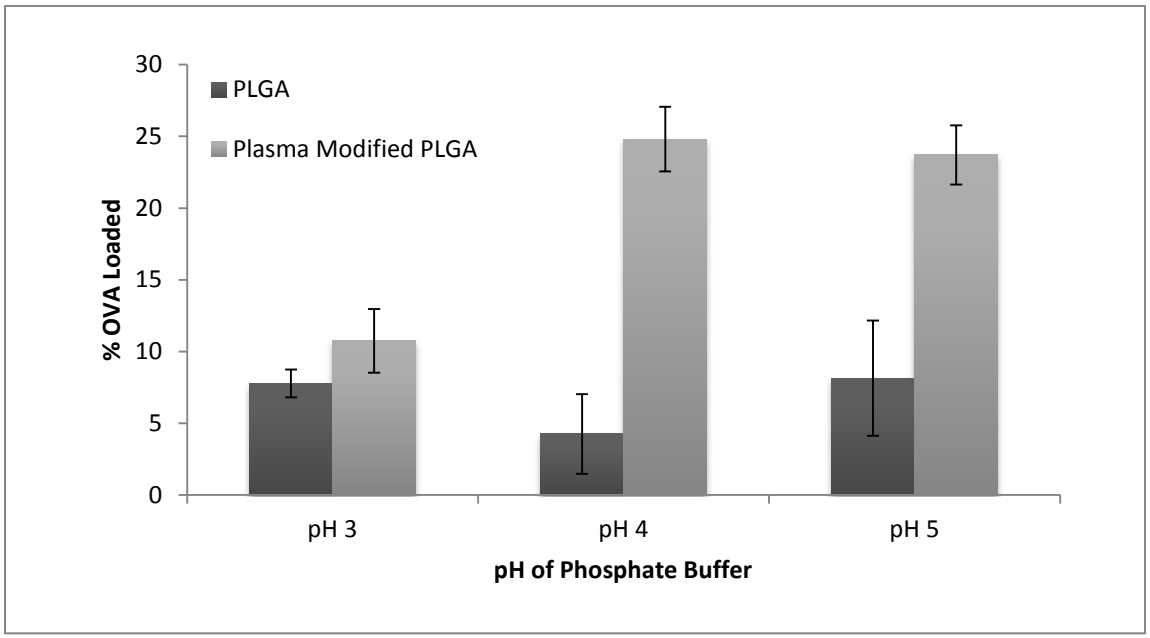

Figure 3.5 Ovalbumin can be loaded onto PLGA microparticles that have been exposed to atmospheric glow discharge plasma for 6 minutes at a frequency of 15 kHz. Protein was loaded at 1.2 w/w% at 4°C overnight in 10 mM phosphate buffer at different pHs. Protein loading was quantified by measuring the non-bound protein using a Micro BCA assay. We were able to obtain a 24% loading efficiency which roughly translates to about 3 μg of ovalbumin per mg of PLGA microparticles.

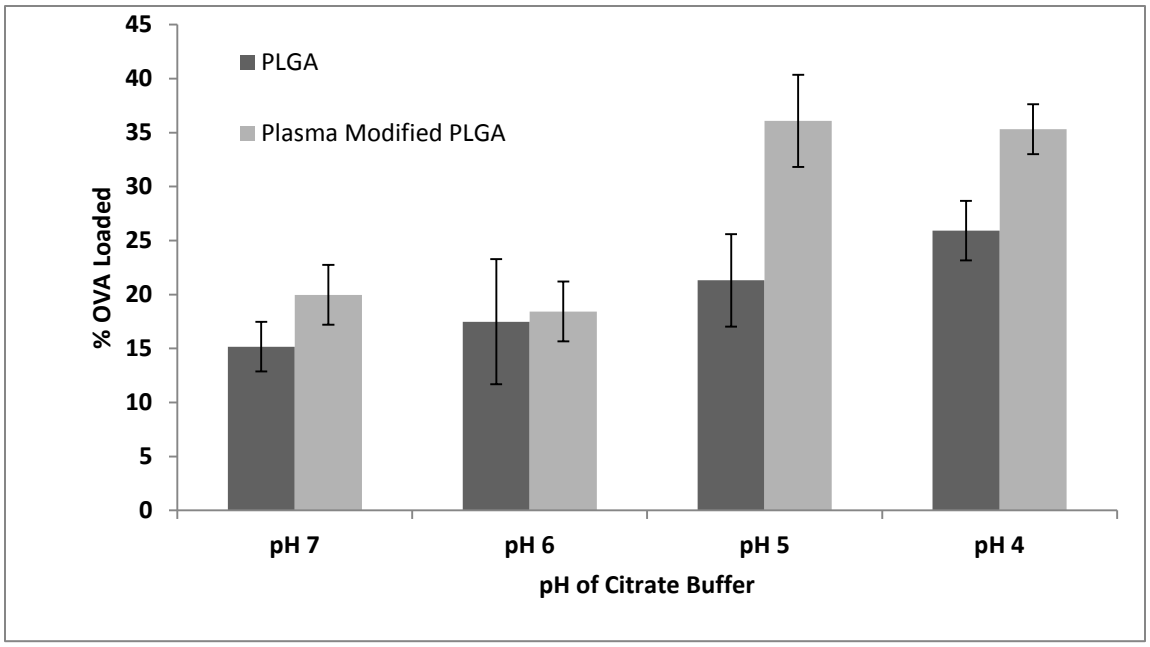

Figure 3.6 Ovalbumin can be loaded onto PLGA microparticles that have been exposed to atmospheric glow discharge plasma for 6 minutes at a frequency of 15 kHz. Protein was loaded at 1.2 w/w% at 4°C overnight in 10 mM citrate buffer. Protein loading was quantified by measuring the non-bound protein using a Micro BCA assay. Under our best loading condition we were able to obtain a 36% loading efficiency which translates to 4.3 µg of ovalbumin per mg of PLGA microparticles.

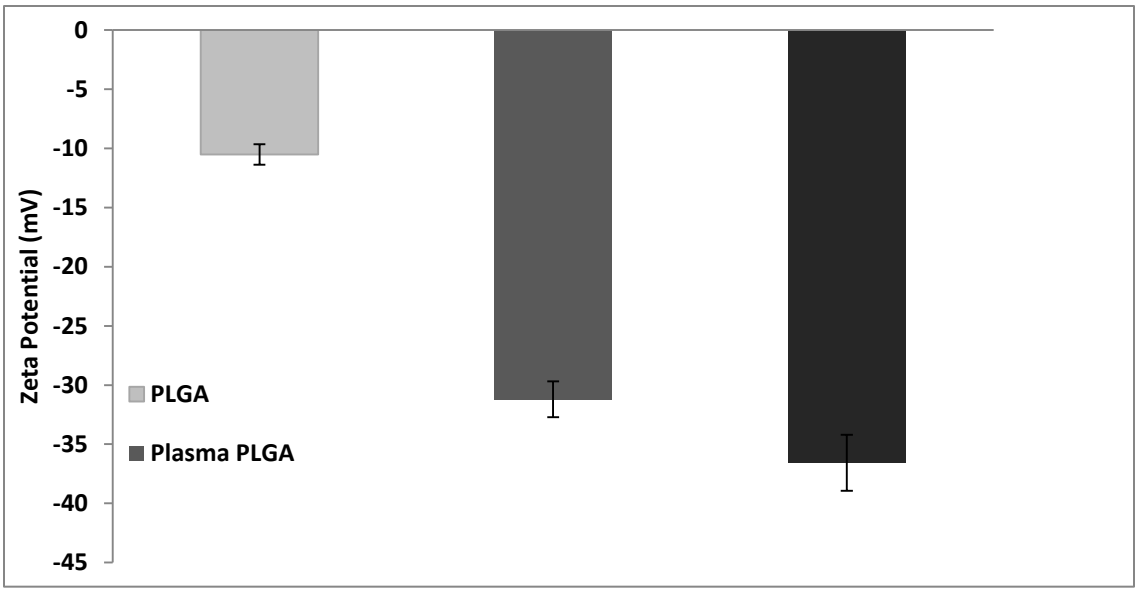

Figure 3.7 We investigated the possibility of long-term storage of our particles by examining the effects that lyopholization may exert on the zeta potential of the particles after plasma modification. No significant difference in zeta potential after lyopholization was seen (as confirmed by analysis using a student's paired t-test).

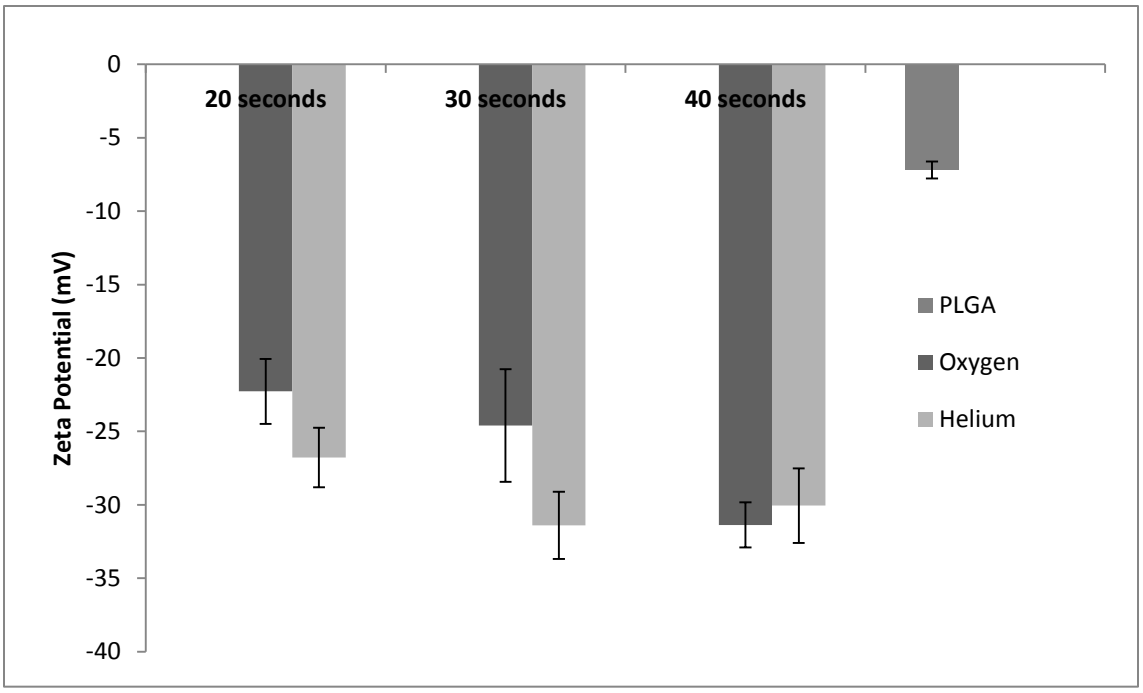

Figure 3.8: Here we investigated the effects of using different gasses and different exposure times on the zeta potential of our PLGA microparticles using a low pressure plasma system.

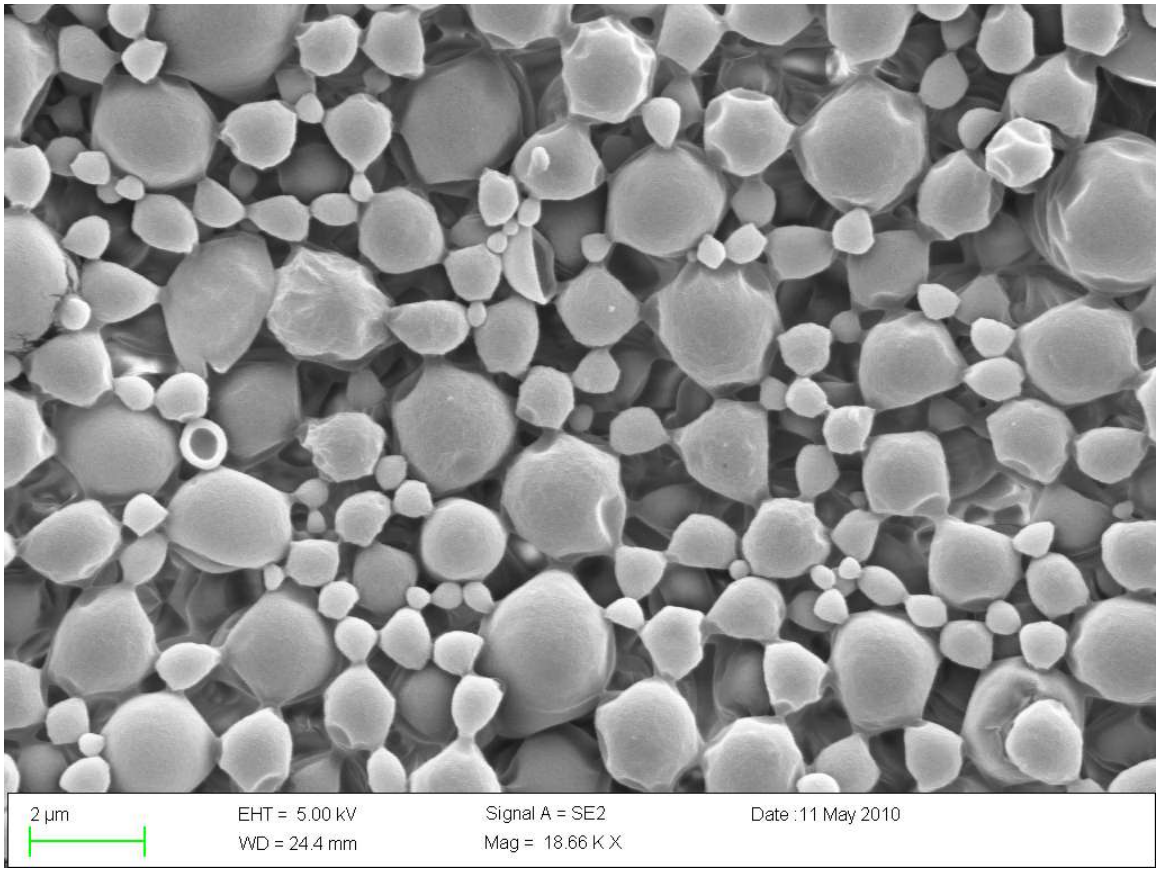

Figure 3.9 Scanning electron micrograph image of un-modified PLGA microparticles reveal particles to have a smooth surface.

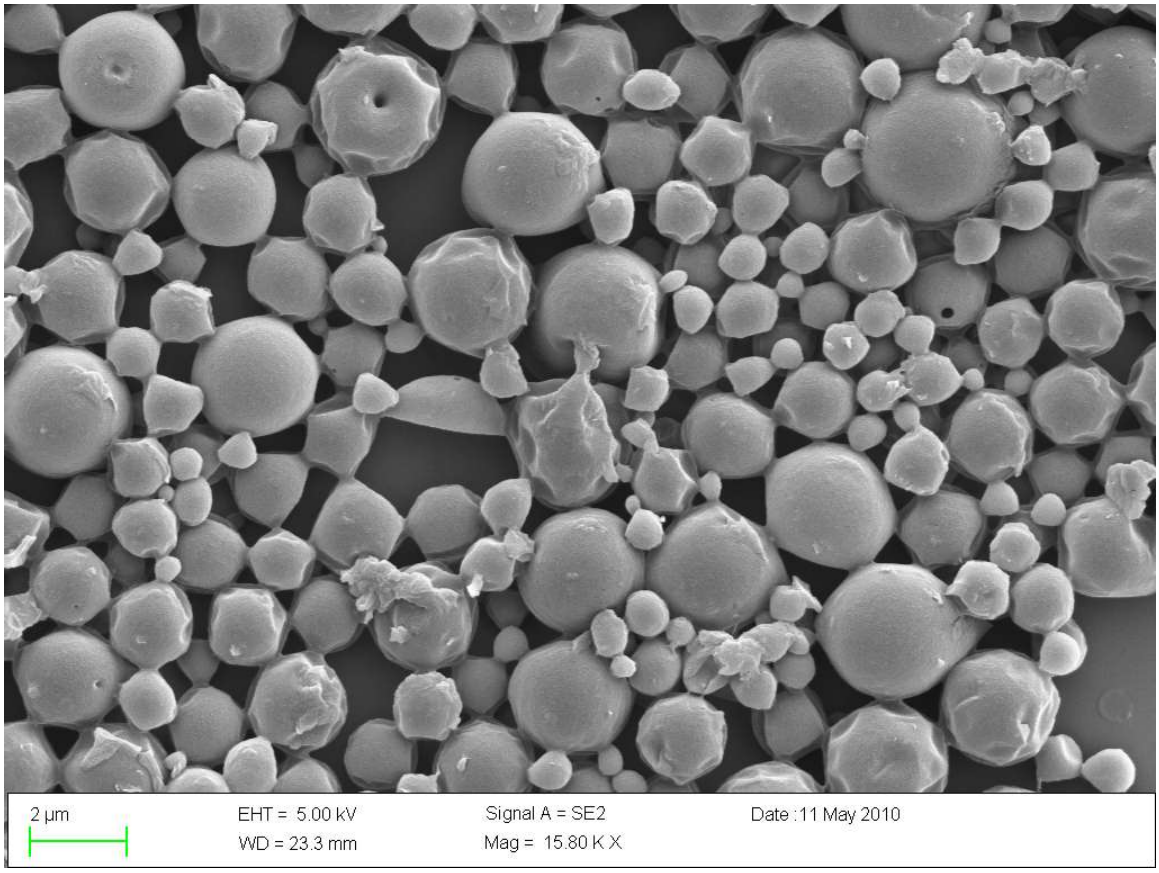

Figure 3.10 Scanning electron micrograph image of low pressure He gas modified PLGA microparticles reveals a slightly rougher surface with signs of possible ablation.

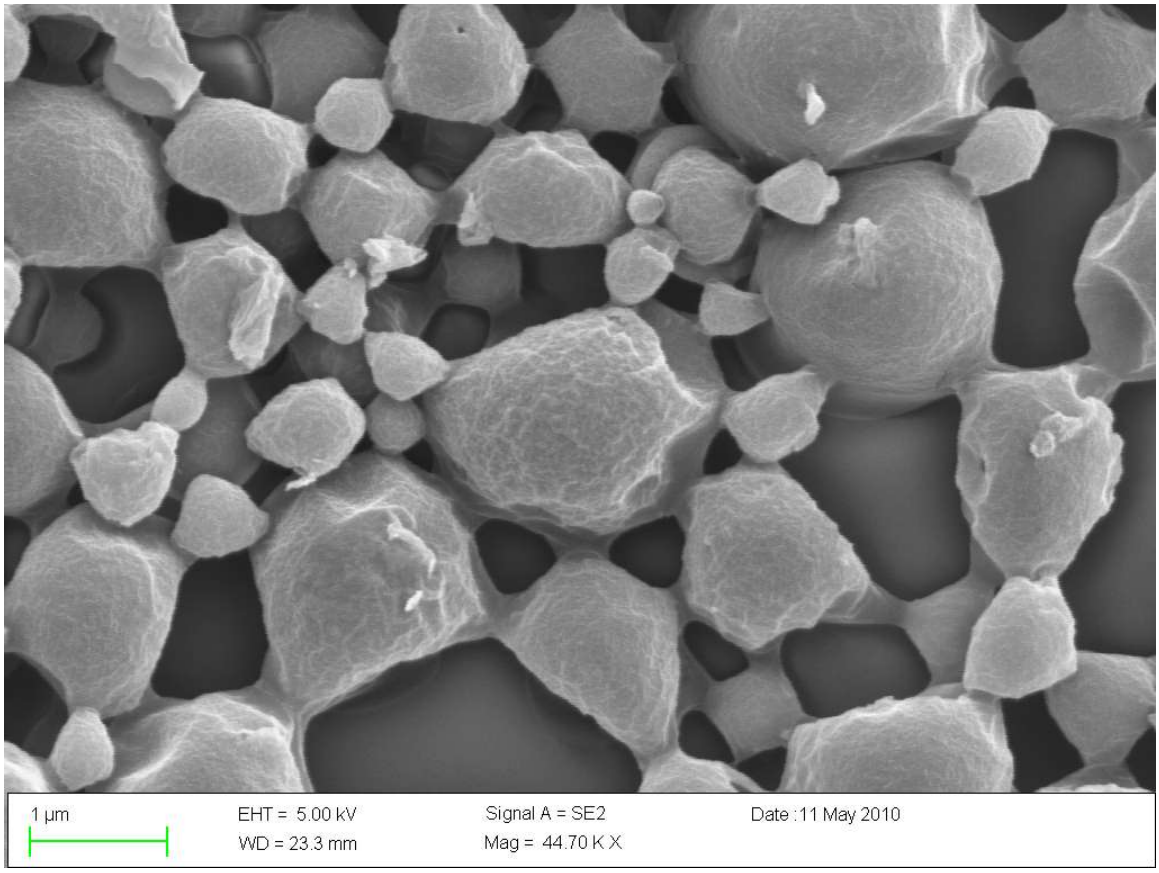

Figure 3.11 A closer image of He low-pressure plasma modified PLGA microparticles reveals a rougher surface.

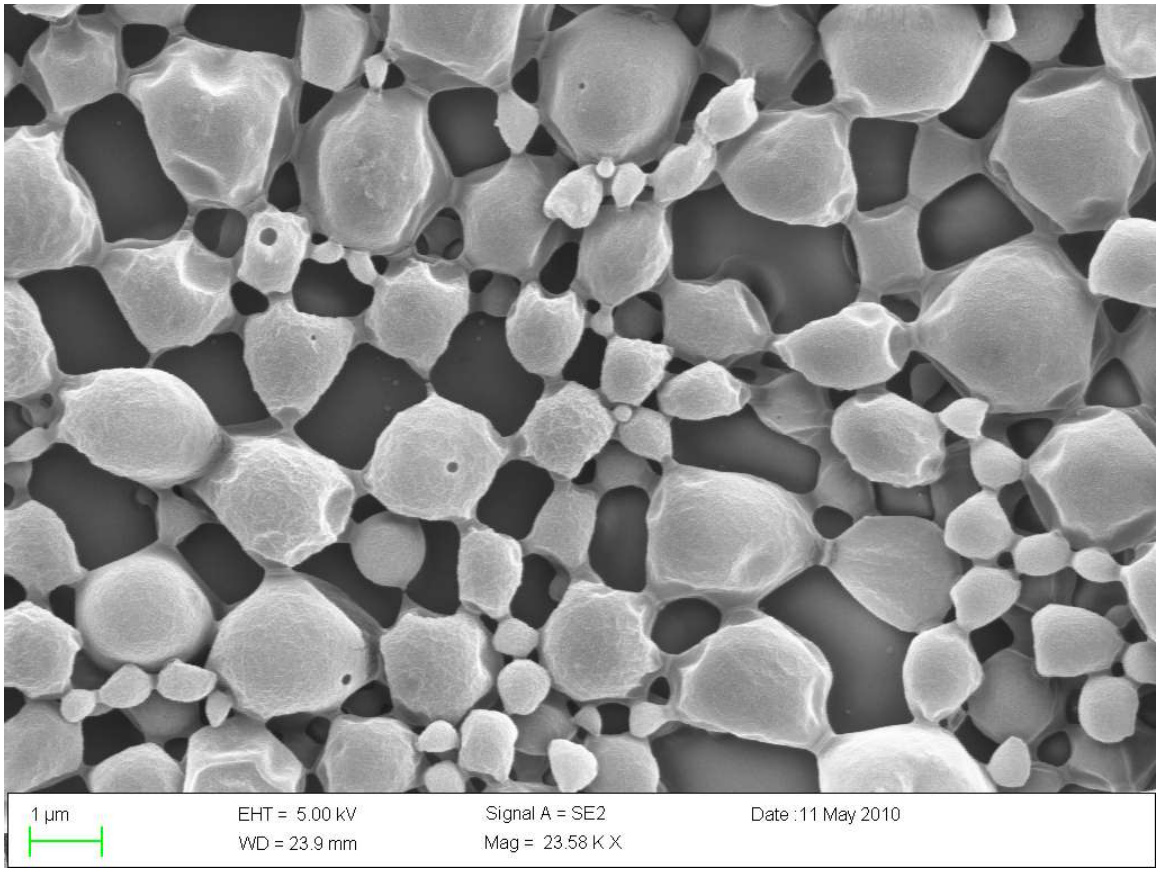

Figure 3.12 Scanning electron micrograph of O_2 low-pressure modified PLGA microparticles shows evidence of a more ablative process, not only in overall surface roughness, but also in actual physical holes created at the surface of these particles

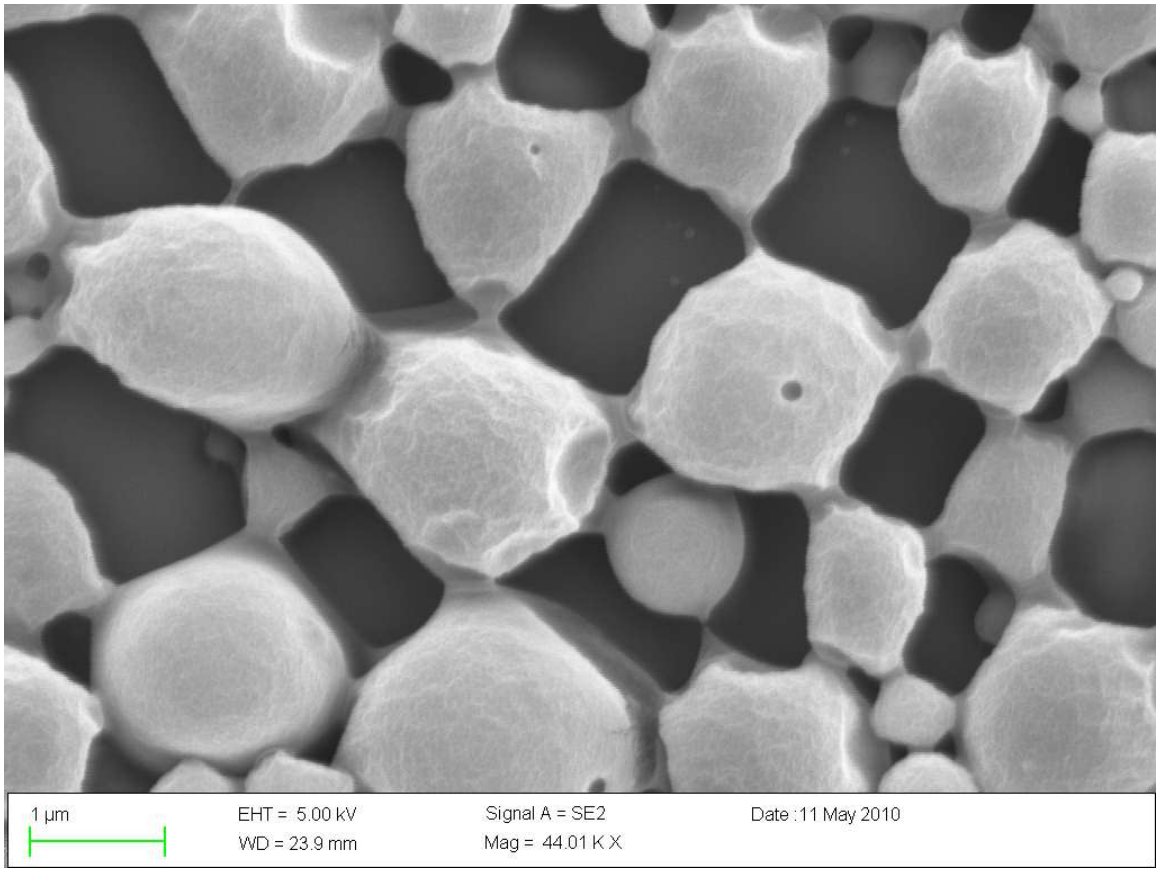

Figure 3.13 A closer scanning electron micrograph image of O_2 low pressure plasma treated microparticles reveals a fairly rough surface with clear holes formed on the surface of these particles.

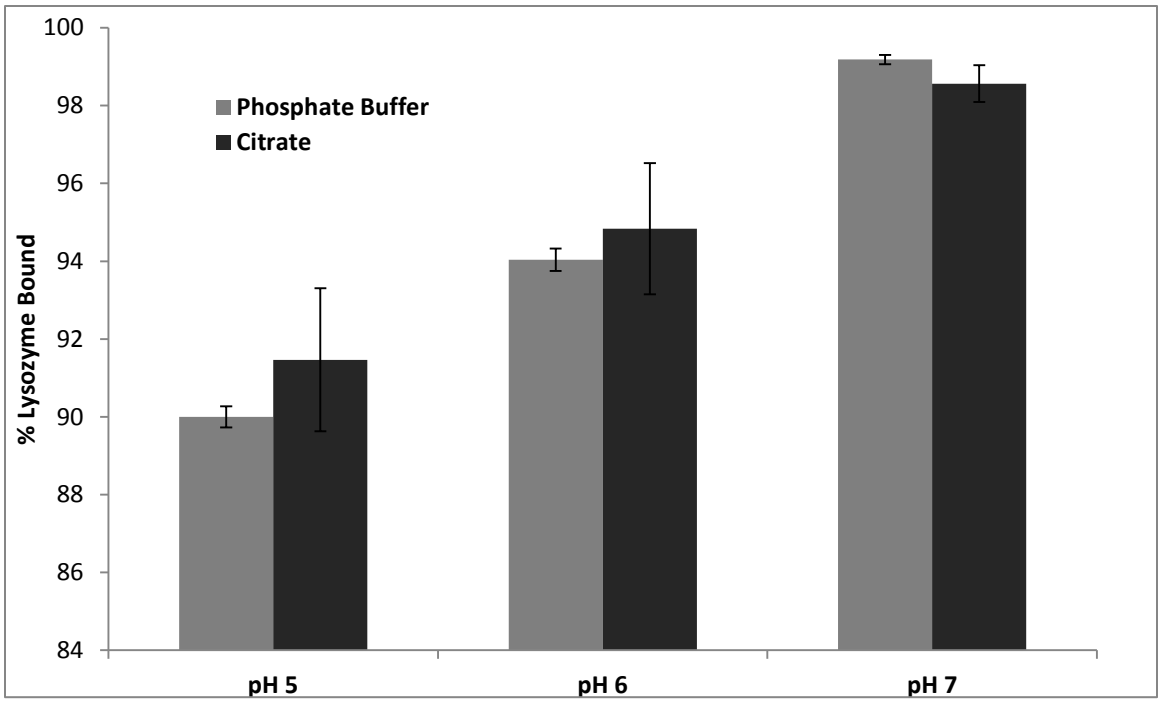

Figure 3.14 Lysozyme, a protein that is cationic at physiological pH binds very efficiently to plasma modified PLGA microparticles.

Table 3.1: Microparticle Surface Relaxes and a significant loss of zeta potential is observed over a short period of time.

Plasma Gas	1 Day	1 Week
O_2	-24.6	-16.0
He	-31.4	-15.9

3.6 REFERENCES

Almeida, A.J., Alpar, H.O., and Brown, M.R.W. (1993). Immune Response to Nasal Delivery of Antigenically Intact Tetanus Toxoid Associated with Poly(l-lactic acid) Microspheres in Rats, Rabbits and Guinea-pigs. Journal of Pharmacy and Pharmacology 45, 198–203.

Ataman- Önal, Y., Munier, S., verine, Gan&eacute, e, A., Terrat, C., line, Durand, P.-Y., et al. (2006). Surfactant-free anionic PLA nanoparticles coated with HIV-1 p24 protein induced enhanced cellular and humoral immune responses in various animal models. Journal of Controlled Release 112, 175–185.

Cui, Z., and Mumper, R.J. (2002). Coating of cationized protein on engineered nanoparticles results in enhanced immune responses. International Journal of Pharmaceutics 238, 229–239.

Desmet, T., Morent, R., De Geyter, N., Leys, C., Schacht, E., and Dubruel, P. (2009). Nonthermal plasma technology as a versatile strategy for polymeric biomaterials surface modification: a review. Biomacromolecules 10, 2351–2378.

Eisenbrey, J.R., Hsu, J., and Wheatley, M.A. (2009). Plasma Sterilization of Poly Lactic Acid Ultrasound Contrast Agents: Surface Modification and Implications for Drug Delivery. Ultrasound in Medicine & Biology 35, 1854–1862.

Huang, Y.-C., Huang, C.-C., Huang, Y.-Y., and Chen, K.-S. (2007). Surface modification and characterization of chitosan or PLGA membrane with laminin by chemical and oxygen plasma treatment for neural regeneration. Journal of Biomedical Materials Research Part A 82A, 842–851.

Ikada, Y. (1994). Surface modification of polymers for medical applications. Biomaterials 15, 725–736.

Kasturi, S.P., Sachaphibulkij, K., and Roy, K. (2005). Covalent conjugation of polyethyleneimine on biodegradable microparticles for delivery of plasmid DNA vaccines. Biomaterials 26, 6375–6385.

Kazzaz, J., Neidleman, J., Singh, M., Ott, G., and O'Hagan, D.T. (2000). Novel anionic microparticles are a potent adjuvant for the induction of cytotoxic T lymphocytes against recombinant p55 gag from HIV-1. J Control Release 67, 347–356.

Laroussi, M. (2005). Low Temperature Plasma-Based Sterilization: Overview and State-of-the-Art. Plasma Processes and Polymers 2, 391–400.

Leleux, J., and Roy, K. (2013). Micro and Nanoparticle-Based Delivery Systems for Vaccine Immunotherapy: An Immunological and Materials Perspective. Advanced Healthcare Materials 2, 72–94.

Mattioli, S., Kenny, J.M., and Armentano, I. (2012). Plasma surface modification of porous PLLA films: Analysis of surface properties and in vitro hydrolytic degradation. Journal of Applied Polymer Science 125, E239–E247.

Morent, R., Geyter, N., Trentesaux, M., Gengembre, L., Dubruel, P., Leys, C., and Payen, E. (2010). Influence of Discharge Atmosphere on the Ageing Behaviour of Plasma-Treated Polylactic Acid. Plasma Chemistry and Plasma Processing 30, 525–536.

Morent, R., De Geyter, N., Desmet, T., Dubruel, P., and Leys, C. (2011). Plasma Surface Modification of Biodegradable Polymers: A Review. Plasma Processes and Polymers 8, 171–190.

Pai Kasturi, S., Qin, H., Thomson, K.S., El-Bereir, S., Cha, S.-C., Neelapu, S., Kwak, L.W., and Roy, K. (2006). Prophylactic anti-tumor effects in a B cell lymphoma model with DNA vaccines delivered on polyethylenimine (PEI) functionalized PLGA microparticles. J Control Release 113, 261–270.

Shin, J., and Raja, L.L. (2003). Dynamics of pulse phenomena in helium dielectric-barrier atmospheric-pressure glow discharges. Journal of Applied Physics 94, 7408–7415.

Shin, J., and Raja, L.L. (2007). Run-to-run variations, asymmetric pulses, and long time-scale transient phenomena in dielectric-barrier atmospheric pressure glow discharges. J. Phys. D: Appl. Phys. 40, 3145.

Singh, A., Nie, H., Ghosn, B., Qin, H., Kwak, L.W., and Roy, K. (2008). Efficient Modulation of T-cell Response by Dual-mode, Single-carrier Delivery of Cytokine-targeted siRNA and DNA Vaccine to Antigen-presenting Cells. Molecular Therapy 16, 2011–2021.

Siow, K.S., Britcher, L., Kumar, S., and Griesser, H.J. (2006). Plasma Methods for the Generation of Chemically Reactive Surfaces for Biomolecule Immobilization and Cell Colonization - A Review. Plasma Processes and Polymers 3, 392–418.

Tendero, C., Tixier, C., Tristant, P., Desmaison, J., and Leprince, P. (2006). Atmospheric pressure plasmas: A review. Spectrochimica Acta Part B: Atomic Spectroscopy 61, 2–30.

Vesel, A., and Mozetic, M. (2012). Surface modification and ageing of PMMA polymer by oxygen plasma treatment. Vacuum 86, 634–637.

Wan, Y., Qu, X., Lu, J., Zhu, C., Wan, L., Yang, J., Bei, J., and Wang, S. (2004). Characterization of surface property of poly(lactide-co-glycolide) after oxygen plasma treatment. Biomaterials 25, 4777–4783.

CHAPTER 4

Cationic PEI Modified PLGA Microparticles: Basic Characterization for Usage as Protein Based Vaccine

4.1 INTRODUCTION

This chapter describes the use of polyethylenimine (PEI) modified PLGA microparticles for the development of a protein based vaccine delivery systems. Microparticles were synthesized in a water in oil in water (w/o/w) emulsion based process and then a simple 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and sulfo N-hydroxysuccinimide (NHS) (EDC/sulfo-NHS) based reaction to functionalize the particles with PEI. Previous work with this system (Singh et al., 2008, 2009) focused on encapsulation of immunomodulatory siRNA and surface loading of pDNA encoding the protein antigen. While this is a promising therapeutic approach, ultimately vaccines utilizing pDNA for protein production have been disappointing in larger primates and humans (as reviewed in (Vajdy et al., 2004)). Part of the limitations of using a pDNA vector to encode for the production of the antigeneic protein are, ultimately, the limitations of cellular transfection: ensuring uptake of here pDNA complexes by the DCs, release of the DNA from endocytotic vesicles into the cytosol, intracellular routing, transport into the nucleus, and ultimately expression of the delivered gene, and, in this case eventual expression onto MHC molecules on the DC. If any of these steps fail, the vaccine will fail to elicit a specific response. We therefor propose using these PEI functionalized microparticles for use in a protein based vaccine. Using electrostatic interactions as our driving force, we then electrostatically attached ovalbumin (OVA), a model protein antigen, to the surface of the synthesized particles. Upon initial *in vitro* characterization, we saw that protein loaded microparticles alone were not sufficient enough to induce strong dendritic cell activation. Again, using

electrostatic interactions, we then attached two known toll-like receptor (TLR) agonists to the surface of the PEI-PLGA microparticles: CpG and siRNA. Initial dose response studies were completed to assess the amount of activation each TLR agonist resulted in. Surface loading of multiple molecules was then assessed for further investigation to examine the effects of loading our protein antigen and our adjuvant on the same particle as compared to separately loaded particles. This data will be discussed in Chapter 5.

4.2 Background and Motivation

Most vaccine formulations function by targeting antigen presenting cells (APCs) such as B cells, macrophages and dendritic cells (DCs), which are known to be the most prominent T-cell activators. As discussed in **Chapter 2**, although DCs have the potential to act as a cellular vaccine many limitations exist in using this type of therapy. Direct and highly efficient *in vivo* delivery of antigens to DCs could overcome the challenges associated with *ex vivo* DC manipulation and may offer a more scalable method for generating immunity.

Bolus injections of purified recombinant proteins or peptides are non-immunogenic when administered without the addition of an adjuvant and are typically subject to degradation and clearance from the body (Bharali et al., 2008). By utilizing recent understandings about the biological nature of how vaccines work to provide long-lasting T cell priming we can work to rationally design an immune response by mimicking the damage signals normally provided by microbial components. The widely used tuberculosis vaccine strain, Mycobacterium bovis BCG, for example, works mainly because it contains a number of TLR agonists including: PG, LAM, lipoproteins, lipopetides, and CpG motifs. A rational combination of appropriate biological cues (PAMPs, chemokines, and cytokines) may be appropriate to a pathology and that a

combination of these could be used to create an optimal immune response specific to the disease (Ehlers and Bulfone-Paus, 2004). Adjuvants can be used to improve the immune response to vaccine antigens in several different ways, and can act by increasing the immunogeneicty of antigens, enhancing the speed and duration of the immune response, modulating antibody avidity, specificity, isotype, or subclass distribution, stimulating CTL, promoting the induction of immunity, enhancing immune responses in immunologically immature or senescent individuals, decreasing the dose of antigen in the vaccine to reduce costs, and helping to overcome antigen competition in combination vaccines (O'Hagan and Singh, 2003; Vajdy et al., 2004).

One way to both enhance protein uptake by APCs as well as be able to co-deliver an immunostimulatory molecule in combination with the protein antigen is by delivering them via particulate systems (Singh et al., 2008, 2004a; Vajdy et al., 2004). As reviewed recently by Leleux and Roy (Leleux and Roy, 2013b) particles used for delivery means serve a number of important purposes namely in that they help to promote the necessary interactions for antigens to be efficiently presented to APCs for both humoral and cellular memory by acting in three important ways: targeting, activation and transfection/antigen presentation. Because particulate delivery systems are more comparable in size to common pathogens (~ 1 um) APCs have been shown to preferentially uptake these particles and therefore the particle associated proteins as well. This enhanced uptake by APCs is an important contributing factor in the ability of these delivery systems to induce more potent immune responses than soluble antigens and, in this way, act as a non-specific targeting mechanism to APCs.

Previous work completed by our group focused on using a particulate based system to deliver pDNA that encoded the protein antigen along with immunomodulatory siRNA specific for IL10 in order to drive a T_H1 type immune response (Kasturi et al.,

2005; Pai Kasturi et al., 2006; Singh et al., 2008, 2009). Kasturi et al. worked extensively on synthesizing and characterizing polyethylene conjugated PLGA microparticles showing that his covalent attachment of branched PEI resulted in particles that offered not only a cationic surface capable of electrostatically attaching pDNA but also buffering capacity that may aid in enhancing phagosomal escape (Kasturi et al., 2005). Here we focus on using his non-encapsulated formulation to load protein based antigens along with other immunomodulatory molecules by utilizing electrostatic interactions.

The objective of this work present here is to show a rational approach to re-purposing a microparticle based system capable of inducing *in vitro* dendritic cell activation that will effectively feed into our next set of *in vitro* and *in vivo* optimization studies. The general experimental set up (illustrated in **Figure 4.1**) demonstrates our basic design, to use our cationic particles to surface load molecules.

4.3 MATERIALS AND METHODS:

4.3.1 Reagents:

PLGA Resomer® RG502H, ovalbumin (used for characterization studies), lysozyme, monosodium phosphate, and disodium phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Poly(vinyl alcohol) PVA MW ~ 31,000 was purchased from Fluka (Sigma-Aldrich, St. Louis, MO). On-Targetplus siRNA for IL10 silencing was purchased from Thermo Fisher Scientific (Waltham, MA), specifically from their Dharmacon subsidiary. We used CpG sequence 1826 (both non-labeled and FITC-CpG) from Invivogen (San Diego, CA). Micro BCA kit for protein analysis was purchased from Thermo Fisher Scientific (Waltham, MA). Silencer Select Cy3-GAPDH siRNA was purchased from Life Technologies, Invitrogen (Austin, TX). Branched PEI

(MW=~70,000 Da) was purchased from Polysciences (Warrington, PA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and sulfo N-hydroxysuccinimide (NHS) were purchased from Pierce Biotechnology (Rockford, IL). Antibodies were purchased from eBioscience (San Deigo, CA) except for CD16/CD32 Fc Block (BD Pharmingen, San Diego, CA). Ovalbumin used for dendritic cell activation studies was Endograde® Ovalbumin and was purchased through BioVendor (Candler, NC). All other lab items were purchased from Fisher Scientific unless otherwise noted.

4.3.2 Primary Dendritic Cell Isolation and Culture:

GM-CSF and IL-4 were purchased from Peprotech (Rock Hill, NJ). Mice were purchased from Jackson or Charles River. Fetal bovine serum was purchased from Hyclone, Thermo Fisher Scientific. Penicillin G with streptomycin was purchased from Invitrogen (Austin, TX). RPMI 1640 was purchased from Sigma Aldrich. Primary APCs will be obtained from bone marrow isolated progenitor cells from Balb/c mice as previously described (Inaba et al., 1992).

Briefly, mice femurs and tibias were be isolated following a protocol approved by the University of Texas at Austin Institutional Animal Care and Use Committee (IACUC). The femurs and tibias of the Balb/C mice were isolated, washed with PBS and, using scissors, both ends of the bones wereremoved and the marrow isolated by flushing out the bone with 2 mL of RPMI 1640 media with a syringe and 25 gauge needles. The collected cells were filtered in order to remove any bone debris. To differentiate the cells into the myeloid lineage, the cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 20 ng/mL mouse GM-CSF and 20 ng/mL IL-4. The media was changed every two days and cells were differentiated for 6-7 days, as

recommended, with the intention of removing non-adherent granulocytes. The percentage of DCs was determined by cell surface marker staining (CD11c) and was typically seen to be ~80%.

4.3.3 PEI functionalized PLGA Microparticle Synthesis:

Microparticles were prepared as described by Kasturi et al and Singh et al (Kasturi et al., 2005; Pai Kasturi et al., 2006; Singh et al., 2008, 2009) using a water-oil-water double emulsion/solvent evaporation technique. Briefly, 0.2 g of PLGA was dissolved in 7 mL of dichloromethane (DCM). 300 uL of deionized water was added to the polymer solution and immediately homogenized for 2 minutes at 10,000 rpm using a Silverson SL2 T homogenizer. The emulsion was then poured into a 1% PVA (Sigma-Aldrich, St. Luis, MD) solution and homogenized for 2 minutes and then the DCM was evaporated from the solution. The microparticles were then collected and washed with deionized water, lyophilized and stored at -20°C.

For bPEI conjugation to PLGA particles: Branched-PEI surface modification of PLGA microparticles was completed as described previously (Kasturi et al., 2005; Pai Kasturi et al., 2006; Singh et al., 2008, 2009). Briefly, 40 mgs of PLGA microparticles were resuspended in 1.35 mL of cold 0.1M MES (2-(N-morpholino) ethane sulfonic acid) buffer (pH 5). EDC (25 molar excess relative to PLGA) and sulfo-NHS ester (40 molar excess relative to PLGA) (Thermo Fisher Scientific Inc, Waltham, MA) was added to the PLGA particle suspension for 2 hours at room temperature. 4 molar excess of bPEI (70 kDa, Polysciences, Washington, PA) was dissolved in 0.2 M MES buffer (pH 6.5) and then the pH was adjusted to 8 and reacted with the activated PLGA microparticles for 2 hours at room temperature. The PEI conjugated microparticles were then collected and

washed 4 times in 1 M NaCl to remove any physically adsorbed PEI and finally once with deionized water. The microparticles were then be lyophilized and stored at -20°C.

4.3.4 Protein and immunomodulatory molecule loading onto PLGA microparticles:

Protein and other immunomodulatory molecules (CpG ODN 1826 (Invivogen, San Diego, CA) and IL10 siRNA (ON-TARGETplus Mouse Il10 siRNA, (Thermo Fisher Scientific, Rockford, IL) are loaded onto our microparticles at 1.2% (wt/wt). Briefly, the molecule being loaded were dissolved in phosphate buffer at the pH being tested. Then, PLGA was suspended in the same loading buffer and added; drop wise, to the dilute solution in low protein binding tubes. The protein-PLGA solution was allowed to incubate overnight on an end to over shaker at 4°C. The particles were then harvested via centrifugation and the supernatant saved for further analysis using a Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and/or NanoDrop Spectrophotometer for single loaded particles. In the case of dual and triple loaded particles, fluorescently labeled immunomodulatory molecules were used. While some background fluorescence was seen between conditions, a standard curve of the second molecule were always run in order to subtract off any possible background. We then subtracted the amount of protein or immunemodulatory molecules found in the supernatant from the amount of protein initially added to our microparticles to determine the amount of protein bound to the microparticle surface. Initial characterization studies were performed using Ovalbumin from Sigma. Microparticles used for cell studies and *in vivo* assays were completed with EndoGrade Ovalbumin purchased from BioVendor (Candler, NC).

4.3.5 Protein release from surface loaded particles:

After particles were loaded, they were spun down and un-bound protein was removed. These samples were re-suspended and incubated at 37°C and placed on an end-over-end shaker. Supernatant was collected at various time points and Micro BCA analysis was performed on the samples.

4.3.6 Surface and size characterization of PLGA microparticles:

Zeta Potential Zeta potential measurement were taken using a Delsa Nano Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Brea, CA) and the Malvern Zetasizer Nano ZS dynamic light scattering instrument as an indirect estimation of surface charge. The presence of PEI modification will be noted by a positive zeta potential as compared to control microparticles. Zeta sizing was taken on Delsa Nano Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Brea, CA).

4.3.7 Dendritic cell *in-vitro* activation:

To ensure that DCs are sufficiently activated by our microparticle formulations we stimulated the immature APCs with our microparticle formulation for 48 hours. Because we were interested in determining the synergistic effects of our microparticles we will investigate the application of a variety of microparticle formulations. These include combinations of: protein loaded and immunomodulatory loaded microparticles as well as microparticles with combinations of both surface loaded proteins as well as immunomodulatory molecules. We then washed the cells with PBS in order to remove microparticles that were not taken up by the APCs. We will then follow a standard FACS staining protocol to stain the cells for appropriate markers. Briefly, cells will be

blocked with anti-mouse CD16/CD32 Fc Block (BD Pharmingen, San Diego, CA) to prevent any nonspecific binding. We then looked at surface expression of CD11c+ DCs of various cell surface markers including CD40, CD86, and CD80. Flow cytometry based analysis will be performed using a BD Accuri flow cytometer and further analysis will be performed using FlowJo software. Cytokine production was also quantified by performing ELISA assays monitoring IL12p40, IL12p70, and IL10 at 6, 24, and 48 hours using ELISA Ready-Set-Go! kits purchased from eBioscience. Data was analyzed using Microsoft Excel and statistical significance was determined.

4.4 RESULTS:

4.4.1 Zeta potential of PLGA microparticles is significantly increased by covalent attachment of PEI

PEI-PLGA microparticles were shown to have a significantly higher zeta potential as compared to their non-functionalized counterparts. This increase was seen at a variety of pHs and further, they demonstrated the largest increase at a pH of 5 (**Figure 4.2**). In KCl solution, PEI-PLGA microparticles typically show a zeta potential of around +32.77while non-functionlalized particles have what would be considered a neutral zeta potential at -6.29.

4.4.2 Ovalbumin loading on PEI-PLGA microparticles

OVA was loaded at high efficiency at a pH of 7. We are capable of achieving a 10.9 μg of protein per mg of PEI-PLGA microparticles. We showed that by optimizing the protein loading conditions (specifically by altering the pH of the loading buffer) we could drive the electrostatic interactions to bind more protein. We also investigated the

usage of citrate buffer (as done in **Chapter 3**) at lower pHs but found that pH of 7 produced the most reliable and highest protein loading.

4.4.3 Protein releases quickly from PEI-PLGA microparticles

We investigated protein release from the PLGA microparticles at 37°C. Here we saw that OVA was released from the particles very quickly, close to 73% was released within the first 24 hours (**Figure 4.4**).

4.4.4 OVA loaded PEI-PLGA microparticles failed to elicit high DC activation

Because DC activation is of the utmost importance, in order to avoid a tollerogenic response to the protein antigen, we were interested in investigating the ability of the particles to induce sufficient DC activation. While an increase in activation was demonstrated (**Figure 4.5**), they were fairly minimal at even higher concentrations. In most data, when using endotoxin-free ovalbumin, typically at a 1 μg/mL concentration, the PEI-PLGA loaded OVA particles failed to elicit a response that was statistically significant from the non-activated DCs.

4.4.5 CpG loading onto PEI-PLGA microparticles

We were able to loadCpG at high loading efficiency onto the surface of our microparticles (Figure 4.6) at lower concentrations (1-2%) but when we attempted to load higher concentrations of CpG (5%) we saw a significant decrease in loading efficiency. We found that, much like the protein loading experiments, by altering pH of the loading particles we could increase loading efficiency, and at a pH of 5. We were able to more

than double our CpG loading capability as compared to the lower dosage we had been investigating previously (**Figure 4.7**).

4.4.6 Higher density of CpG on microparticles does not induce significantly higher expression of activation markers on DCs

We investigated if loading higher quantities of CpG on a microparticle would induce higher amounts of activation in DCs. When comparing the same overall concentration of CpG (2 μg/mL) we saw no significant increase in DC activation markers between the lower and higher loaded microparticles (**Figure 4.8**). When we compared conditions based off of the mgs of PEI-PLGA delivered, we did see a slight increase in activation marker expression.

4.4.7 Increasing concentrations of TLR agonists increases DC activation surface marker expression

We demonstrated that by increasing, in a step-wise manner, the concentration of TLR agonists (**Figure 4.9** and **Figure 4.10**) we saw a similar step-wise increase in cell surface marker expression. Here we see that CpG molecules resulted in a much higher increase of activation markers (**Figure 4.10**) as compared to siRNA (**Figure 4.9**).

4.4.8 PEI-PLGA micropartcles are capable of loading multiple immunomoedulatory molecules

Next, we investigated our particles' ability to load multiple immunostimulatory molecules in order to ensure that we were delivering multiple molecules to a single cell. For dual delivery particles, we focused on OVA, the protein antigen, and CpG (**Figure 4.11**). The dual delivery system was based off of data from section 4.4.7 where CpG

resulted in higher amounts of activation, as compared to siRNA. We then took our work further demonstrating that we were capable of loading three stimulatory molecules (siRNA, CpG, and protein) onto a single particle (**Figure 4.12**) at high efficiency. For dual loaded particles, we were capable of binding 11.4 μg of OVA and 11.9 μg of CpG onto a single particle. For the triple loaded particles we demonstrated an ability to load 10.2 μg of OVA, 9.72 μg of CpG and 9.72 μ of siRNA. All molecules were loaded at a concentration of 1.2 wt% (so for dual particles that's a total of 2.4 wt% of molecules and 3.6% for triple loaded particles).

4.5 DISCUSSION

Despite significant progress in immunotherapy based research, infectious diseases and cancer continue to be a significant world-wide health burden. New immunization strategies need to be employed, however, in order to generate effective immune responses to chronic viral or tumor antigens (most of which are self-antigens) as reviewed recently by Friede and Aguado (Friede and Aguado, 2005). While many of the most potent vaccines on today's market are given as a live attenuated or killed form of the particulate microorganism, there are numerous potential drawbacks for both of these strategies (as reviewed in **Chapter 2**). These include poor efficacy of boosting antibody responses and safety concerns. By applying recent immunological findings such as new information about the cells of the immune system and the microenvironments within which these cells reside to vaccine development, it may be possible to create a safer, more cost effective vaccination.

In this chapter we presented basic characterization and a rational design for a vaccine strategy that we will evaluate further in Chapter 5. As reviewed in **Chapter 2**, many groups have investigated the usage of charged particles to deliver protein antigens

adsorbed onto the surface of particulate based systems. In doing so, groups have shown a high ability to control the amount and disposition of proteins through the use of buffers, and have been able to optimize the protein loading process depending on the particulate system (Lamalle-Bernard et al., 2006; O'Hagan and Singh, 2003; Singh et al., 2004a, 2004b). Here we took our particulate system and systematically altered loading conditions to maximize protein loading of our anionic model antigen. We showed that under optimized conditions we were capable of eliciting high amounts of protein loading (**Figure 4.3**). Further, we showed that the protein releases quickly from our microparticle system (**Figure 4.4**) which may act to prevent some of the deleterious effects observed with antigen-depot systems (Hailemichael et al., 2013). However, the particulate delivery system did not induce high amounts of DC activation (Figure 4.5). For this reason we began to investigate combining our protein delivering molecules along with an adjuvant. Initially we focused on using CpG 1826, a commonly used adjuvant (as reviewed briefly in **Chapter 2**). Particles loaded with CpG induced significantly higher amounts of activation (**Figure 4.8**) on their own and in combination with other particles (**Figure 4.10**). We further looked to functionalize our system by including siRNA specific to IL-10. Using siRNA for IL-10 has been shown to reduce IL-10 production, result in an increase in CD40 expression (after DC activation), and increased Th1 response *in-vitro* (Liu et al., 2004). Further, when siRNA for IL-10 is transfected along with another TLR agonist (Poly(I:C)) it acts synergistically with the other adjuvant to cause an enhanced Th1 anti-tumor response (Akasaki et al., 2011). We therefore decided to continue the investigations with the functional usage of dual and triple loaded PEI-PLGA microparticles.

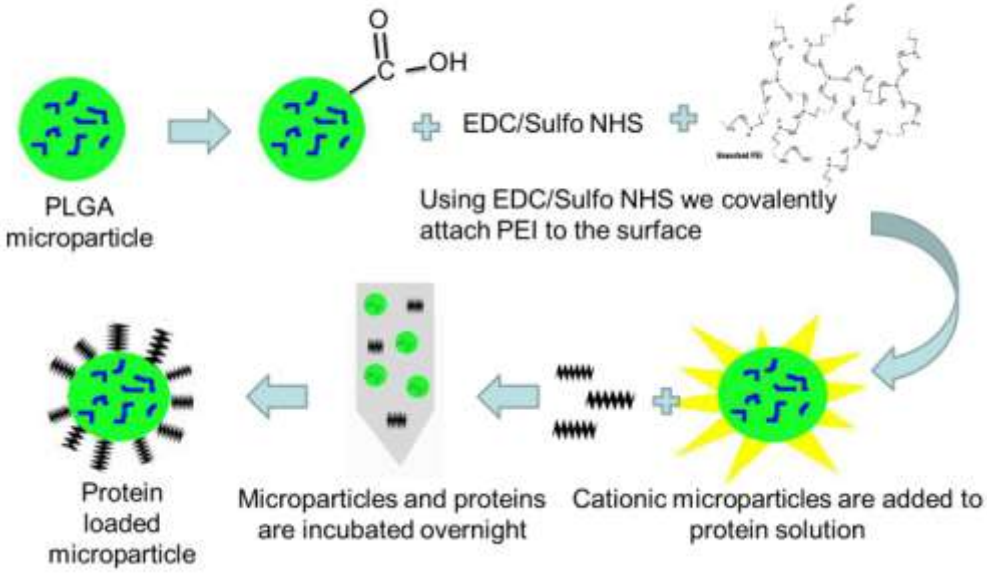

Figure 4.1 Schematic representation of PEI protein loading experiments. PLGA microparticles are made using an w/o/w emulsion process. The PLGA used is acid end capped allowing us to use simple EDC/NHS chemistry to covalently attach branched PEI to the surface of our particles. We can further functionalize our PEI-PLGA by electostatically attach protein (or other anionic materials) to the surface of our cationic particles.

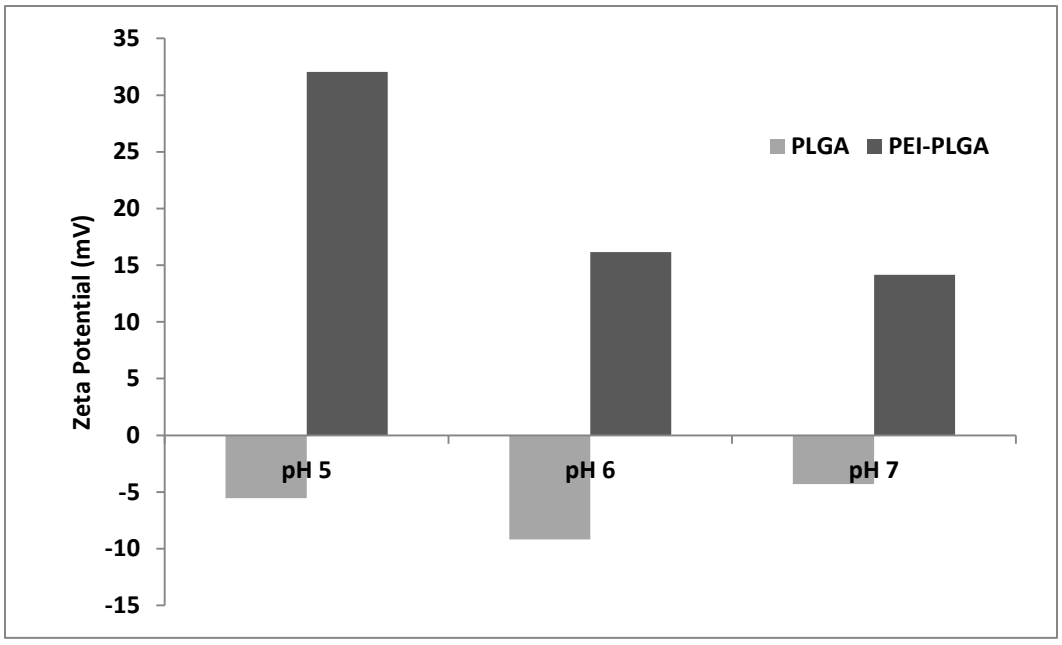

Figure 4.2 Zeta potential analysis of PEI-PLG and PLGA microparticles in phosphate buffer. Here we investigated the effect of pH on the zeta potential of our materials. Briefly, we suspended our particles at 4% w/v in 10 mM phosphate buffer.

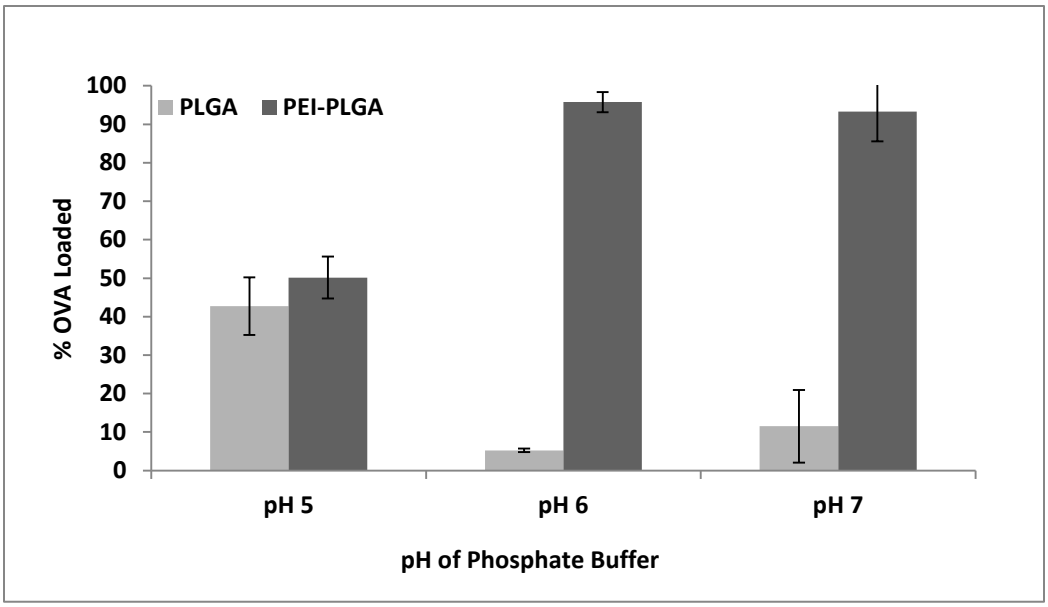

Figure 4.3 PEI-PLGA microparticles are capable of loading significant amounts of ovalbumin: Ovalbumin was loaded onto microparticles in a dilute solution (1.2 w/w%) of PBS (10 mM) at various pHs. Maximum protein loading was observed at pH of 7 and was equivalent to 10.9 μg/mg of PEI-PLGA.

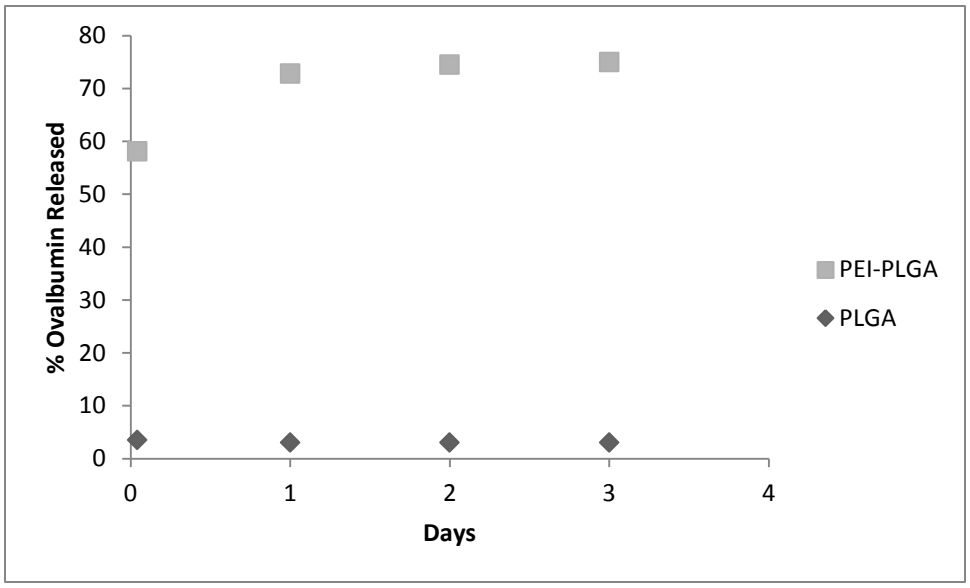

Figure 4.4 Ovalbumin release from PEI-PLGA microparticles at 37°C: After protein loading, particles were incubated on an end-over-end shaker at 37°C to assess the amount of protein released from the particles. Here we see that within the first 24 hours about 73% of protein loaded was released.

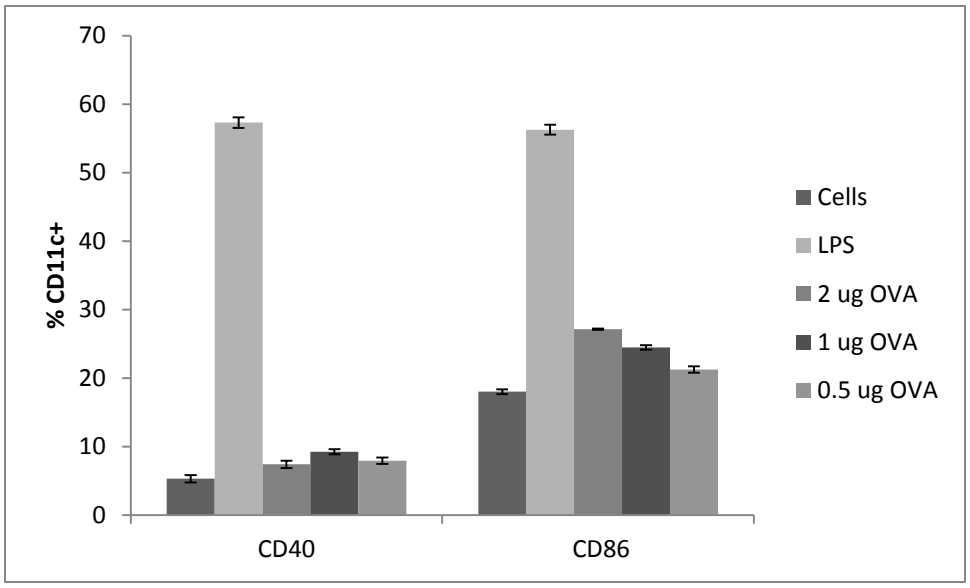

Figure 4.5 PEI-PLGA microparticles loaded with ovalbumin did not induce high amounts of stimulatory surface marker expression on primary DCs: Because, as mentioned previously, activation of DCs is of the utmost importance, we assessed whether our particles could induce DC activation. Here we show very little amounts of co-stimulatory marker production, indicating the necessity of adding an adjuvant to our formulation.

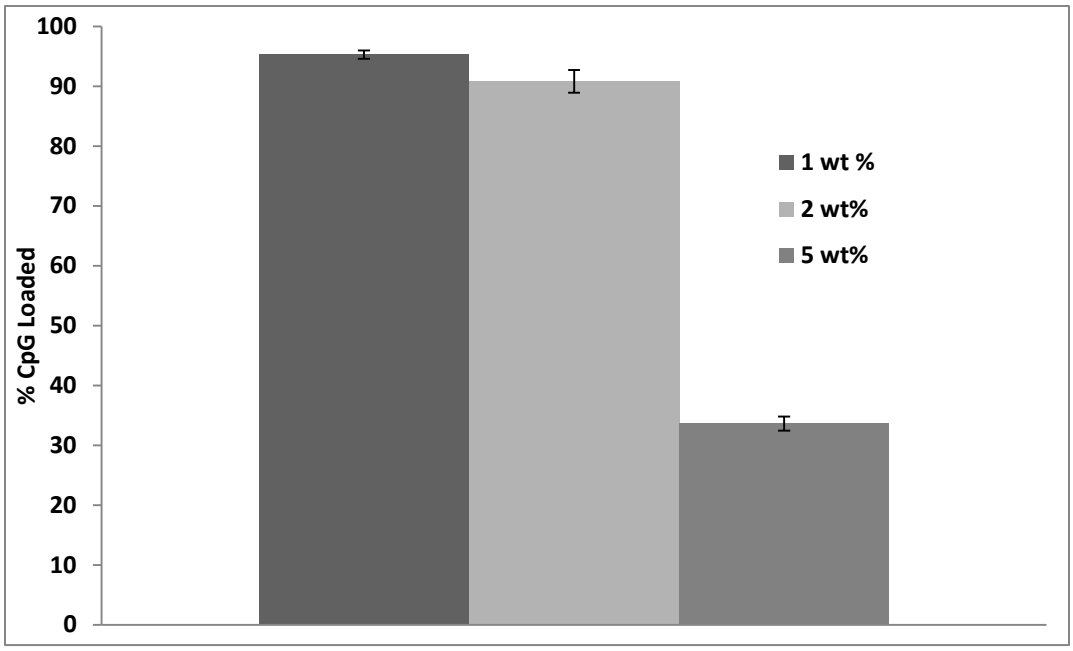

Figure 4.6 CpG loading onto PEI-PLGA microparticles: We investigated the loading capabilities of PEI-PLGA microparticles to surface load small CpG sequences. We performed these investigations at a pH of 6 (based off of previous work loading pDNA onto the surface of PEI-PLGA microparticles (Kasturi et al., 2005).

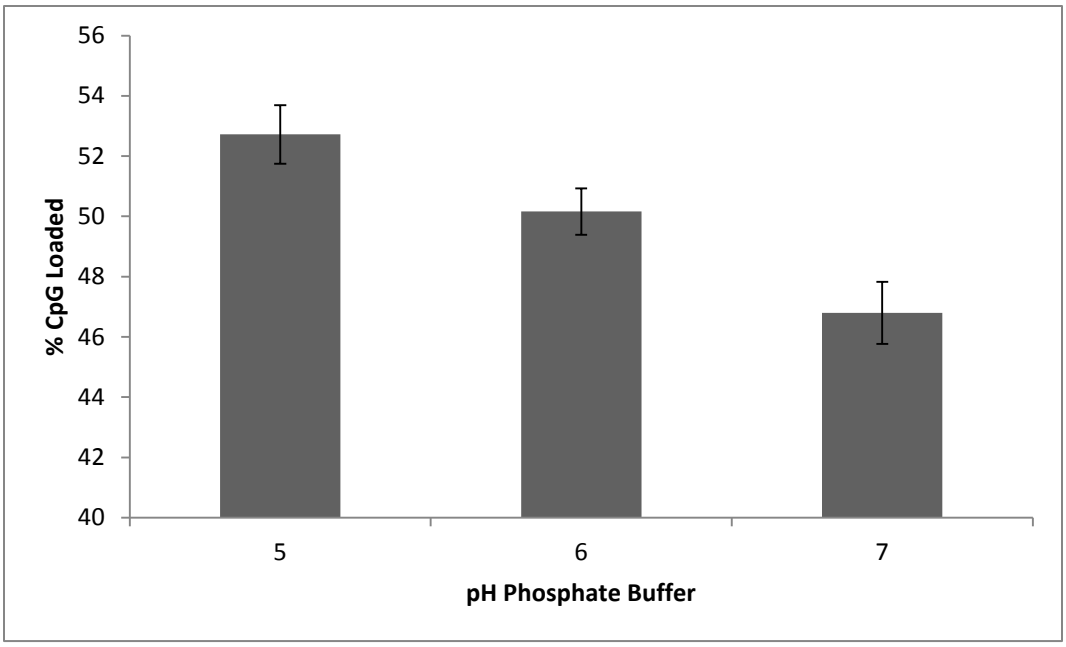

Figure 4.7 Altering the pH of the loading buffer enables us to increase the load of CpG on the surface of PEI-PLGA microparticles: By adjusting the pH of the phosphate buffer we were able to increase the CpG loading (at 5 w/w%) where at optimal conditions the loading was equivalent to approximately 26 µg/mg (as compared to 1.2 w/w% loading which was seen to load 9.5 µg/mg).

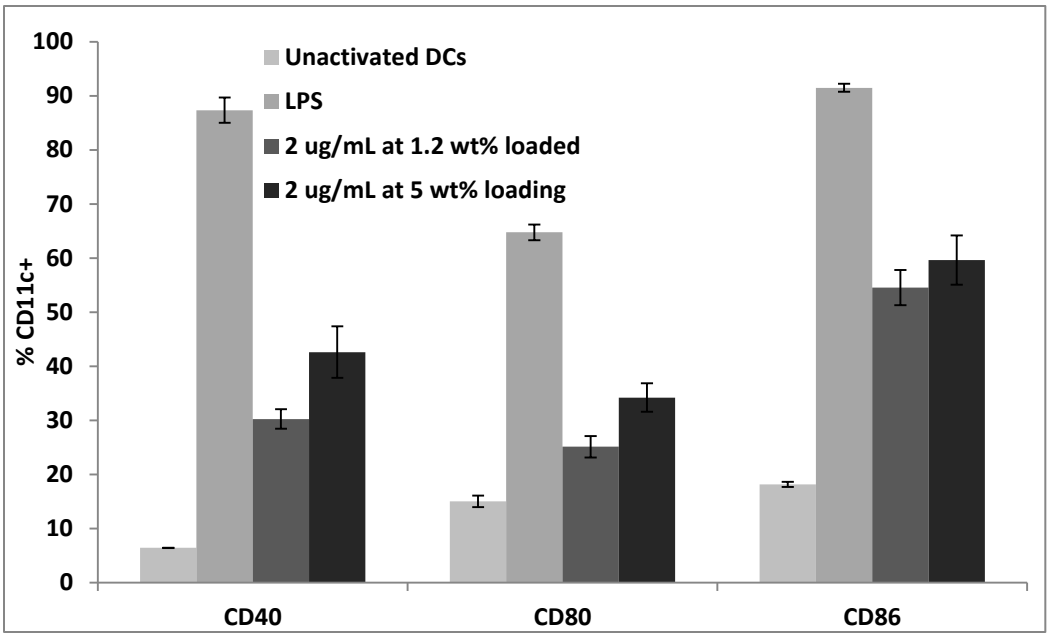

Figure 4.8 Dendritic cell surface activation marker expression in cells stimulated with CpG loaded PEI-PLGA microparticles: Dendritic cells were activated with CpG loaded microparticles and cell surface expression of co-stimulatory molecules was assessed. Although higher densities of CpG did elicit a slightly higher expression of stimulatory markers, the increase was not significant.

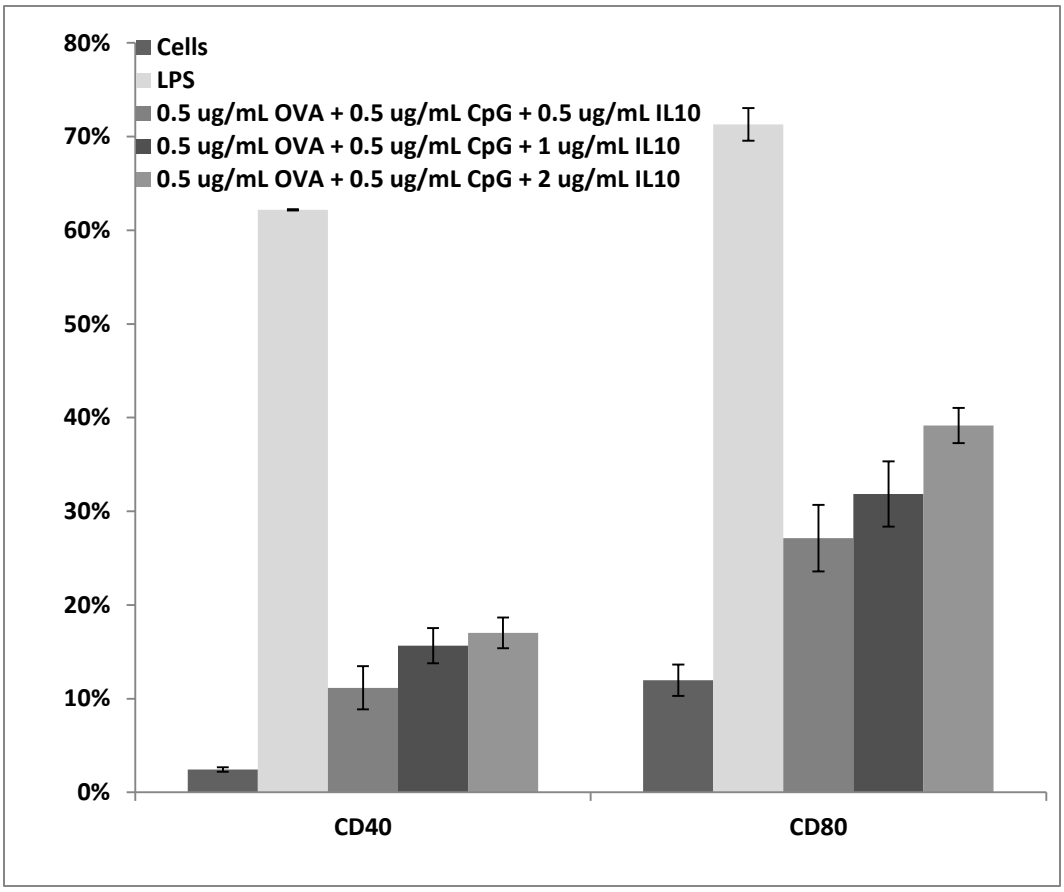

Figure 4.9 Dendritic cell activation increased by increasing dosage of siRNA for IL10: To determine the effects of altering a known TLR agonist, we systematically altered the dosage of siRNA loaded microparticles while keeping lower dosages of ovalbumin (the model protein antigen) and CpG (a second TLR agonist). We were looking to determine the synergistic effects of the three particles delivered. Here we see that as siRNA loaded particles are increased, you can see an increase in cell surface activation markers.

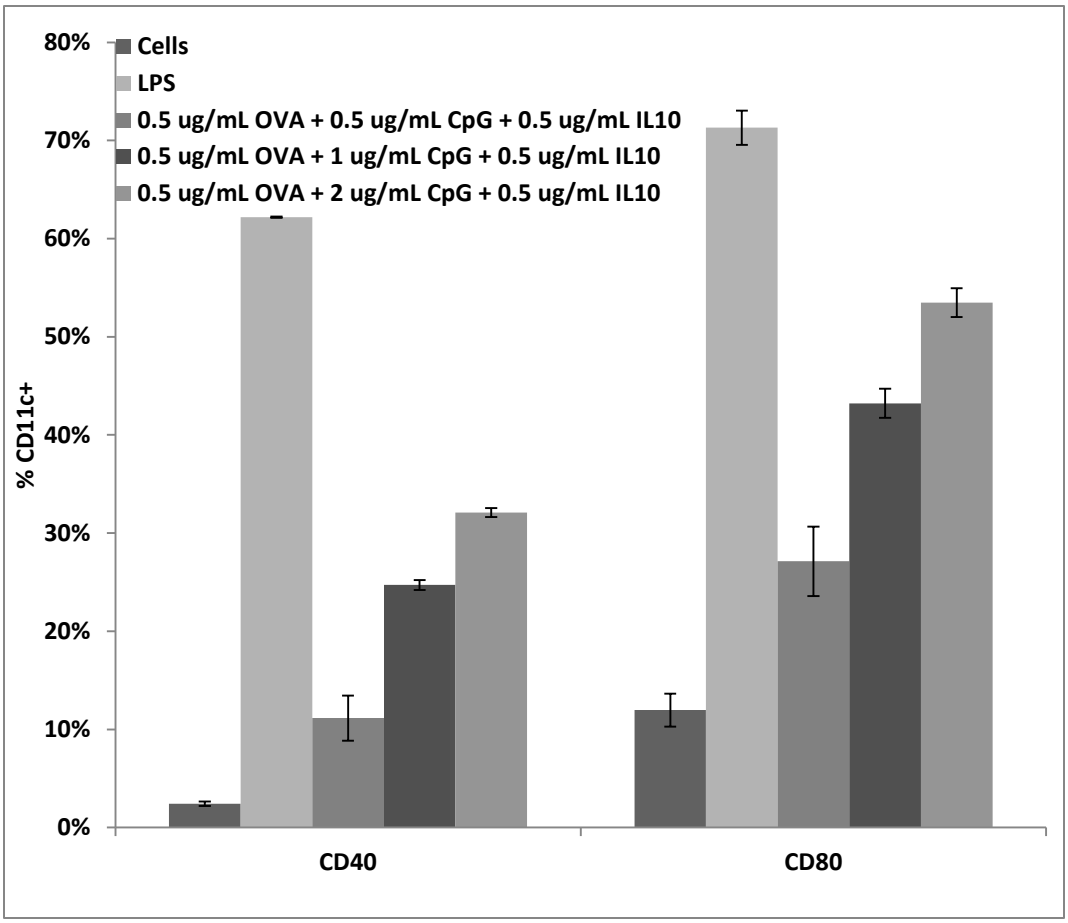

Figure 4.10 Increasing CpG loaded PEI-PLGA microparticles cause a significant increase in activation markers in DCs: Here, keeping lower dosages of our model protein antigen (ovalbumin) loaded PEI-PLGA microparticles and siRNA specific for IL10 PEI-PLGA microparticles, we systematically increased the dosage of CpG loaded PEI-PLGA microparticles.

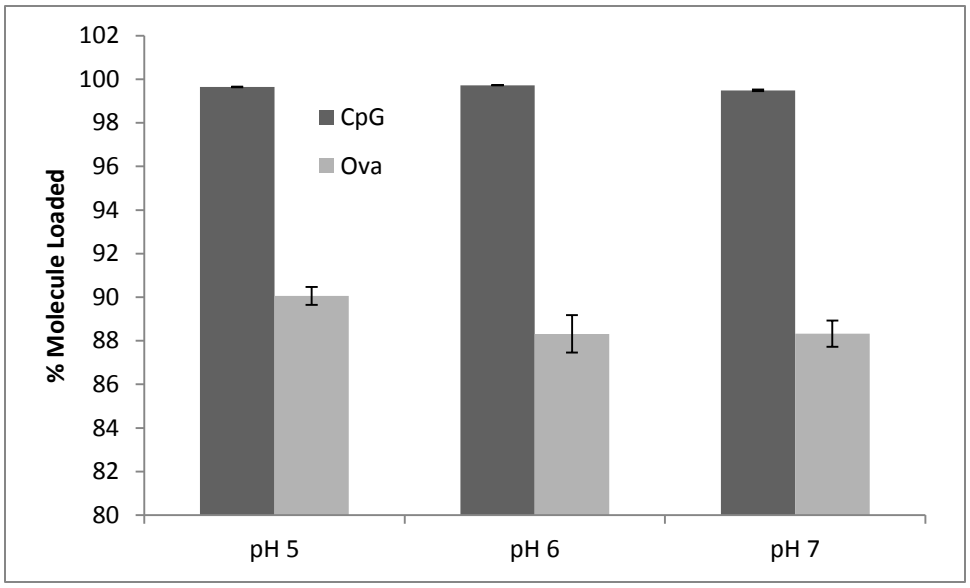

Figure 4.11 PEI-PLGA microparticles are capable of binding both CpG and ovalbumin: Here we demonstrate our particles ability to load both CpG and ovalbumin in order to co-deliver both an adjuvant particle and a protein on the same particle. Here we used fluorescently labeled CpG (FITC) to simply measure fluorescence of the supernatant (i.e. unbound) CpG. To measure ovalbumin loading, similarly, we measured the amount of unbound ovalbumin in the supernatant of our samples and calculated from that what was actually bound to the particles. Here, under best conditions, we demonstrated an ability to deliver 11.4 µg of OVA and 11.9 µg of CpG per mg of PEI-PLGA microparticle.

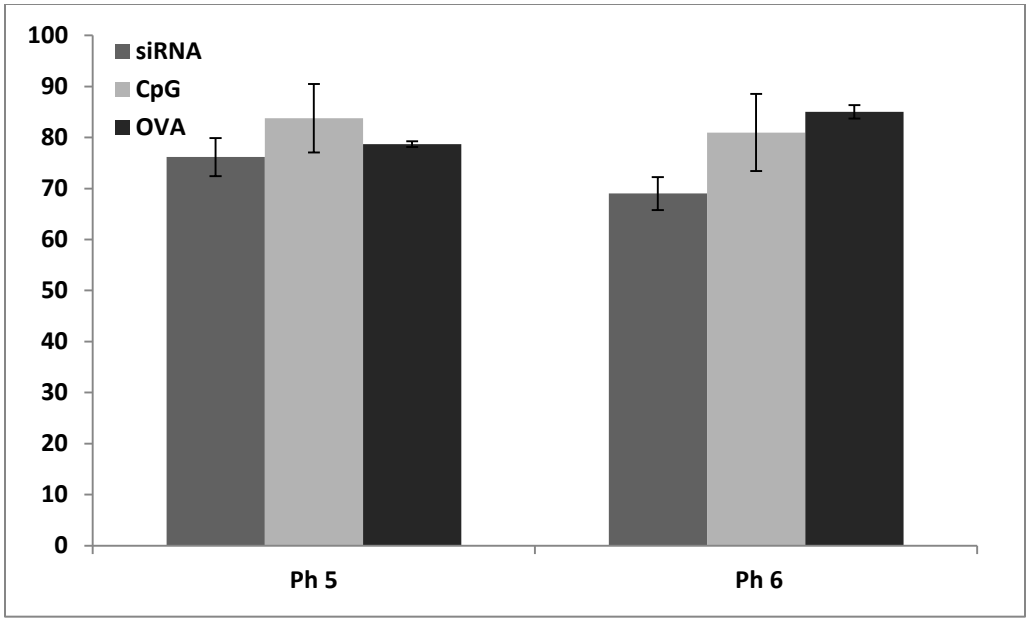

Figure 4.12 PEI-PLGA microparticles can efficiently load three separate molecules (CpG, siRNA for IL10 and a model protein, ovalbumin): Here we demonstrated the ability to load 10.2 µg of ovalbumin, 8.28 µg of siRNA, and 9.72 µg of CpG per mg of PEI-PLGA. CpG and siRNA were quantified using fluorescence where we subtracted any background fluorescence reading from the other stimulatory molecule. Protein quantification was completed using a micro BCA assay. All molecules were loaded at 1.2 wt% (so in total 3.6 wt% of molecules were added to our PEI-PLGA microparticles).

4.6 REFERENCES

Akasaki, Y., Kikuchi, T., Irie, M., Yamamoto, Y., Arai, T., Tanaka, T., Joki, T., and Abe, T. (2011). Cotransfection of Poly(I. Journal of Immunotherapy 0, 121–128.

Bharali, D.J., Pradhan, V., Elkin, G., Qi, W., Hutson, A., Mousa, S.A., and Thanavala, Y. (2008). Novel nanoparticles for the delivery of recombinant hepatitis B vaccine. Nanomedicine: Nanotechnology, Biology and Medicine 4, 311–317.

Ehlers, S., and Bulfone-Paus, S. (2004). Shaping Adaptive Immunity against Pathogens: The Contribution of Innate Immune Responses. In Novel Vaccination Strategies, S.H.E. Kaufmann, ed. (Wiley-VCH Verlag GmbH & Co. KGaA), pp. 17–50.

Friede, M., and Aguado, M.T. (2005). Need for new vaccine formulations and potential of particulate antigen and DNA delivery systems. Adv. Drug Deliv. Rev. 57, 325–331.

Hailemichael, Y., Dai, Z., Jaffarzad, N., Ye, Y., Medina, M.A., Huang, X.-F., Dorta-Estremera, S.M., Greeley, N.R., Nitti, G., Peng, W., et al. (2013). Persistent antigen at vaccination sites induces tumor-specific CD8+ T cell sequestration, dysfunction and deletion. Nat Med 19, 465–472.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 176, 1693–1702.

Kasturi, S.P., Sachaphibulkij, K., and Roy, K. (2005). Covalent conjugation of polyethyleneimine on biodegradable microparticles for delivery of plasmid DNA vaccines. Biomaterials 26, 6375–6385.

Lamalle-Bernard, D., Munier, S., Compagnon, C., Charles, M.-H., Kalyanaraman, V.S., Delair, T., Verrier, B., and Ataman-Önal, Y. (2006). Coadsorption of HIV-1 p24 and gp120 proteins to surfactant-free anionic PLA nanoparticles preserves antigenicity and immunogenicity. Journal of Controlled Release 115, 57–67.

Leleux, J., and Roy, K. (2013). Micro and Nanoparticle-Based Delivery Systems for Vaccine Immunotherapy: An Immunological and Materials Perspective. Advanced Healthcare Materials 2, 72–94.

Liu, G., Ng, H., Akasaki, Y., Yuan, X., Ehtesham, M., Yin, D., Black, K.L., and Yu, J.S. (2004). Small interference RNA modulation of IL-10 in human monocyte-derived dendritic cells enhances the Th1 response. European Journal of Immunology 34, 1680–1687.

O'Hagan, D.T., and Singh, M. (2003). Microparticles as vaccine adjuvants and delivery systems. Expert Rev Vaccines 2, 269–283.

Pai Kasturi, S., Qin, H., Thomson, K.S., El-Bereir, S., Cha, S.-C., Neelapu, S., Kwak, L.W., and Roy, K. (2006). Prophylactic anti-tumor effects in a B cell lymphoma

model with DNA vaccines delivered on polyethylenimine (PEI) functionalized PLGA microparticles. J Control Release 113, 261–270.

Singh, A., Nie, H., Ghosn, B., Qin, H., Kwak, L.W., and Roy, K. (2008). Efficient Modulation of T-cell Response by Dual-mode, Single-carrier Delivery of Cytokine-targeted siRNA and DNA Vaccine to Antigen-presenting Cells. Molecular Therapy 16, 2011–2021.

Singh, A., Suri, S., and Roy, K. (2009). In-situ crosslinking hydrogels for combinatorial delivery of chemokines and siRNA-DNA carrying microparticles to dendritic cells. Biomaterials 30, 5187.

Singh, M., Kazzaz, J., Chesko, J., Soenawan, E., Ugozzoli, M., Giuliani, M., Pizza, M., Rappouli, R., and O'Hagan, D.T. (2004a). Anionic microparticles are a potent delivery system for recombinant antigens from Neisseria meningitidis serotype B. Journal of Pharmaceutical Sciences 93, 273–282.

Singh, M., Chesko, J., Kazzaz, J., Ugozzoli, M., Kan, E., Srivastava, I., and O'Hagan, D.T. (2004b). Adsorption of a novel recombinant glycoprotein from HIV (Env gp120dV2 SF162) to anionic PLG microparticles retains the structural integrity of the protein, whereas encapsulation in PLG microparticles does not. Pharm. Res. 21, 2148–2152.

Vajdy, M., Srivastava, I., Polo, J., Donnelly, J., O'Hagan, D., and Singh, M. (2004). Mucosal adjuvants and delivery systems for protein-, DNA- and RNA-based vaccines. Immunology and Cell Biology 82, 617–627.

CHAPTER 5

PEI-PLGA microparticles for *in-vitro* and *in-vivo* dendritic cell activation towards the rational design of a protein based vaccine

5.1 INTRODUCTION:

This chapter explores the usage of the optimized microparticle based system investigated in **Chapter 4** in both *in vitro* and *in vivo* models. We investigated both single and combinatorial loaded microparticles for usage as a platform protein vaccine delivery system. Here we show that we can efficiently activate dendritic cells (DCs) *in vitro* by investigating their surface expression of activation markers as well as cytokine secretion of T_H1 associated cytokines as well as increases in gene expression in response to our particulate formulations. From these *in vitro* assays we chose an optimized particulate system for further investigations, specifically to study the effects of delivering our protein antigen on the same particle as an adjuvant vs. separate delivery. We next studied the effects of our microparticles *in vivo* in an ovalbumin expressing melanoma tumor model where we investigated its potential in both a therapeutic and prophylactic model. Here we showed that our microparticles could reduce tumor growth and significantly increased survival. In our *in vivo* models, we found that for phophylactic studies the dual delivered protein and adjuvant performed statistically better than separate protein and adjuvant delivery. For therapeutic usage, there was no statistically significant difference between dual vs. separate delivery.

5.2 BACKGROUND AND MOTIVATION

As discussed previously, proteins themselves are very weakly immunogenic and when delivered without the addition of an adjuvant incapable of eliciting an immune response. As reviewed recently by Perrie et al (Perrie et al., 2008) there is no standard,

general recipe for what makes an effective vaccine, and instead, many criteria exist for any given vaccine to function properly. One of the main concerns is that the vaccine generates the appropriate immune response desired against that specific antigen. As discussed in Chapter 2, effective vaccines often include a variety of different immune-stimulatory molecules that work in combination to achieve the desired effects (Ehlers and Bulfone-Paus, 2004). By focusing on the disease state and understanding the progression of the disease, it may be possible to rationally design the desired reaction.

An imbalance between the T_H1 and T_H2 response has been implicated in several chronic infectious diseases such as human immunodeficiency virus infection and chronic hepatitis B and C infections and cancer. Mature T helper cells, also referred to as CD4+ T cells, are central to the induction of anti-viral responses and have been subdivided according to two predominant cytokine secretion profiles. T_H1 cells produce cytokines such as interleukin (IL)-2, interferon-gamma (IFN-γ), and tumor necrosis factor beta which are important factors responsible for promoting the cell-mediated immune response. T_H2 cells produce cytokines such as IL-4, IL-5 and IL-10 which mediate the humoral, or antibody mediated response. Cytokines released by one type of T_H lymphocyte population can down-regulate the functions of the other T_H population subset. Furthermore, it has been suggested that the initial development of APCs into T_H1 or T_H2 phenotype is believed to depend on the leading cytokines at the site of initial antigen presentation, the type of antigen presenting cell, the nature of the co-stimulatory molecules involved, and the dose of stimulatory antigens. This phenotypic commitment may be critical for the subsequent differentiation of immune cells nonspecifically recruited at the site of infection (as reviewed in (Thompson, 1995)).

By delivering multiple adjuvants using a single particle system it may be possible to take advantage of multiple pathways to synergistically control the immune response to a given antigen (Hermans et al., 2007; Salvador et al., 2012).

5.3 MATERIALS AND METHODS:

5.3.1 Reagents

PLGA Resomer® RG502H, ovalbumin (used for characterization studies), lysozyme, monosodium phosphate, and disodium phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Poly(vinyl alcohol) PVA MW ~ 31,000 was purchased from Fluka (Sigma-Aldrich, St. Louis, MO). On-Targetplus siRNA for IL10 silencing was purchased from Thermo Fisher Scientific (Waltham, MA), specifically from their Dharmacon subsidiary. We used CpG sequence 1826 (both non-labeled and FITC-CpG) from Invivogen (San Diego, CA). Micro BCA kit for protein analysis was purchased from Thermo Fisher Scientific (Waltham, MA). Silencer Select Cy3-GAPDH siRNA was purchased from Ambion, Invitrogen (Austin, TX). Branched PEI (MW=~70,000 Da) was purchased from Polysciences (Warrington, PA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and sulfo N-hydroxysuccinimide (NHS) were purchased from Pierce Biotechnology (Rockford, IL). Antibodies and ELISA Ready-Set-Go!® Elisa kits were purchased from eBioscience (San Deigo, CA) except for CD16/CD32 Fc Block (BD Pharmingen, San Diego, CA). Ovalbumin used for dendritic cell activation studies was Endograde® Ovalbumin and was purchased through BioVendor (Candler, NC). RNAeasy kit and RT² SYBR® green qPCR mastermix was purchased from Qiagen (Hamburg, Germany). SuperScript® III First-Strand Synthesis System was purchased from Invitrogen (Carlsbad, CA). Primers

for RT-PCR for IL12p40, IL12p35, IL10, and house keeping gene mouse β-actin was purchased from SABiosciences (Valencia, CA). All other lab items were purchased from Fisher Scientific unless otherwise noted.

5.3.2 Primary Dendritic Cell Isolation and Culture:

GM-CSF and IL-4 were purchased from Peprotech (Rock Hill, NJ). Mice were purchased from Jackson or Charles River. Fetal bovine serum was purchased from Hyclone, Thermo Fisher Scientific. Penicillin G with streptomycin was purchased from Invitrogen (Austin, TX). RPMI 1640 was purchased from Sigma Aldrich. Primary APCs were obtained from bone marrow isolated progenitor cells from Balb/c mice as previously described (Inaba et al., 1992). Briefly, mice femurs and tibias were isolated following a protocol approved by the University of Texas at Austin Institutional Animal Care and Use Committee. The femurs and tibias of the Balb/C mice were isolated, washed with PBS and, using scissors, both ends of the bones were removed and the marrow isolated by flushing out the bone with 2 mL of RPMI 1640 media with a syringe and 25 gauge needle. The collected cells were filtered in order to remove any bone debris. To differentiate the cells into the myeloid lineage, the cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 20 ng/mL mouse GM-CSF and 20 ng/mL IL-4. The media was changed every two days and cells were differentiated for 6-7 days, as recommended, with the intention of removing non-adherent granulocytes. The percentage of DCs was determined by cell surface marker staining (CD11c) and was typically seen to be ~80%.

5.3.3 Synthesis of PLGA microparticles

Microparticles were prepared as described by Kasturi et al and Singh et al(Kasturi et al., 2005; Pai Kasturi et al., 2006; Singh et al., 2008, 2009) and further in **Chapter 3 and 4**. Briefly, PLGA (RG502H, Evonik Rohm GmbH, Essen, Germany) was dissolved in dichloromethane (DCM) (Thermo Fischer Scientific Inc., Waltham, MA). Deionized water was added to the polymer solution and immediately homogenized and then the first emulsion was poured into a 1% PVA (Sigma-Aldrich, St. Luis, MD) solution and homogenized and then the DCM was evaporated from the solution. The microparticles were then collected and washed with deionized water, lyophilized and stored at -20°C.

5.3.4 bPEI conjugation to PLGA particles

Branched-PEI surface modification of PLGA microparticles was completed as described previously (Kasturi et al., 2005; Pai Kasturi et al., 2006; Singh et al., 2008, 2009) and in detail in **Chapter 4**. Briefly, PLGA microparticles were resuspended in 0.1M MES (2-(N-morpholino) ethane sulfonic acid) buffer (pH 5). EDC and sulfo-NHS ester (Thermo Fisher Scientific Inc, Waltham, MA) was added to the PLGA particle suspension for 2 hours at room temperature. bPEI (70 kDa, Polysciences, Washington, PA) was dissolved in 0.2 M MES buffer (pH 6.5) and then the pH was adjusted to 8 and reacted with the activated PLGA microparticles for 2 hours at room temperature. The PEI conjugated microparticles were then collected and washed 4 times in 1 M NaCl to remove any physically adsorbed PEI and finally once with deionized water. The microparticles were then be lyophilized and stored at -20°C.

5.3.5 Protein and immunomodulatory molecule loading onto PLGA microparticles

Protein and other immunomodulatory molecules (CpG ODN 1826 (Invivogen, San Diego, CA) and IL10 siRNA (ON-TARGETplus Mouse Il10 siRNA, Thermo Scientific, Rockford, IL) were loaded onto our microparticles at 1.2% (wt/wt). As described in **Chapter 4**, protein was dissolved into the appropriate loading buffer: PBS pH 7 for OVA alone, PBS pH 6 for OVA+CpG, PBS pH 6 for OVA+CpG+siRNA, PBS pH 5 for CpG, PBS pH 5 for siRNA. Then, PLGA was dissolved in the same loading buffer and was added; drop wise, to the dilute solution in low protein binding tubes. The protein-PLGA solution was allowed to incubate overnight on an end to over shaker at 4°C. The particles were then harvested via centrifugation and the supernatant saved for further analysis using a Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and/or NanoDrop Spectrophotometer. We then subtracted the amount of protein or immunemodulatory molecules found in the supernatant from the amount of protein initially added to our microparticles to determine the amount of protein bound to the microparticle surface. Initial characterization studies were performed using Ovalbumin from Sigma. Microparticles used for cell studies and in vivo assays were completed with EndoGrade Ovalbumin purchased from BioVendor.

5.3.6 Characterization of PLGA microparticles

Zeta potential measurements were taken using a Delsa Nano Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Brea, CA) and the Malvern Zetasizer Nano ZS dynamic light scattering instrument as an indirect estimation of surface charge. The presence of PEI modification was noted by a positive zeta potential as compared to control microparticles. Zeta sizing was taken on Delsa Nano Zeta Potential and Submicron Particle Size Analyzer (Beckman Coulter, Brea, CA).

5.3.7 *In vitro* Characterization

Primary APCs were obtained from bone marrow isolated progenitor cells from Balb/c mice as previously described (Inaba et al., 1992) and previously in section **4.3.2**.

To ensure that our microparticles are sufficiently activated by our microparticle formulations we stimulated the immature APCs with our microparticle formulation for 48 hours. Because we were interested in determining the synergistic effects of our microparticles we investigated the application of a variety of microparticle formulations. These included combinations of: OVA protein loaded and immunomodulatory-molecule (CpG and siRNA for IL10) loaded microparticles as well as microparticles with combinations of both surface loaded OVA protein as well as immunomodulatory molecules (specifically OVA and CpG loaded dual microparticles and OVA, CpG, and siRNA triple loaded microparticles). We then washed our cells with PBS in order to remove microparticles that were not taken up by the APCs. We then followed a standard FACS staining protocol to stain our cells for appropriate markers. Briefly, cells were blocked with anti-mouse CD16/CD32 Fc Block (BD Pharmingen, San Diego, CA) to prevent any nonspecific binding. We then looked at surface expression of CD11c+ DCs of various cell surface markers including CD40, CD86, and CD80. Flow cytometry based analysis was performed using a BD Accuri flow cytometer and further analysis was performed using FlowJo software. Cytokine production was also quantified by performing ELISA assays monitoring IL12p40, IL12p70, and IL10 at 6, 24, and 48 hours using ELISA Ready-Set-Go!® Elisa kits purchased from eBioscience. Briefly, plates were prepared and coated with the appropriate capture antibody at 4°C overnight. Plates were washed and standards and samples were incubated for 2 hours at room temperature.

After washing, detection antibody was added and incubated at room temperature for an additional hour. Washing was repeated and finally Avidin-HRP was added for detection purposes. Unbound Avidin-HRP was removed via washing and the plate was developed using the provided substrate solution and read at 450 nm. Data was analyzed using Microsoft Excel and statistical significance was determined. RT-PCR was also quantified using the same microparticle formulations. Briefly, RNA was extracted from BMDCs using RNAeasy kit (Qiagen, USA). Complementary DNA (cDNA) was synthesized from total RNA using SuperScript® III First-Strand Synthesis System (Invitrogen, CA), and real time RT-PCR was performed using RT² SYBR® green qPCR mastermix (Qiagen, Germany) on an Applied Biosystem 7900HT Fast real time PCR system. Specific primers for mouse IL12p40, IL12p35, IL10, and house keeping gene mouse β-actin (SABiosciences, CA) as reference gene were used. Relative gene expression level for the target gene were calculated following ΔΔCT method using the formula: Target gene expression of sample = $2(-\Delta\Delta CT)$, where $\Delta\Delta CT$ = [CT (target gene) − CT (reference gene)] of treatment − [CT (target gene) − CT (reference gene)] of control and the threshold cycle (CT) is the PCR cycle at which first signal of reporter fluorescence above a baseline signal is detected.

5.3.8 *In vivo* tumor model challenge: prophylactic and therapeutic models

All in vivo studies were performed following a protocol approved by the University of Texas at Austin Institutional Animal Care and Use Committee. Two models were investigated in C57BL/6 mice: a prophylactic model as well as a therapeutic model. For the prophylactic model, briefly, mice were given injections all

subcutaneously of the following formulations: PBS, IFA and Ovalbumin, Ovalbumin loaded microparticles, Ovalbumin loaded microparticles with CpG loaded microparticles, Ovalbumin and CpG loaded microparticles. Dosage of all formulations were kept constant: each mouse received a dosage of 50 μg/mouse of OVA and 50 μg/mouse of CpG. A total of three injections were given and on day 30 B16/OVA melanoma cells were injected ($1*10^5$ cells/mouse). Tumor growth was measured using a digital caliper from Mitutoyo. When tumors reached critical size (400 mm^3) or ulcerated mice were sacrificed. In the therapeutic model mice were given an injection of $5*10^5$ cells and then injections of microparticle formulations on days 4, 11, and 18. Again, tumor growth was monitored and mice were sacrificed when tumors reached a critical size of 400 mm^3 or when the tumor ulcerated. Statistical analysis was performed using SPSS software.

5.4 RESULTS

5.4.1 *In vitro* characterization and dendritic cell activation

In order to fully characterize and optimize our particle formulations, extensive in vitro studies using primary dendritic cells were performed. Loading levels of all biological molecules were established prior to *in vitro* characterization studies (**Table 1** and **Figure 5.1**) and particle dosage was normalized to loading levels for all studies. All results shown are normalized to a concentration of 1 μg of OVA (the model antigen) delivered per well (24 well plate at 500,000 cells per well). Cell surface marker expression was analyzed as described (**Figure 5.2** and **Figure 5.3**). Soluble OVA failed to elicit cell surface activation makers and were not significantly different as compared to non-activated dendritic cells. All particulate conditions as well as conditions containing CpG (soluble or particle associated) elicited a significant increase in cellular activation

markers while soluble siRNA failed to illicit a statistically significant response in surface marker expression. In all conditions, activation was significantly higher when immunomoledulatory molecules were delivered using particles as compared to their soluble forms. In most conditions, no significant difference was observed between dual loaded particles and triple loaded particles.

Cytokine secretion by activated dendritic cells was examined at 6, 24, and 48 hour time points for IL12p70, IL12p40, and IL10 (**Figure 5.5**, **Figure 5.6**, **Figure 5.7**, and **Figure 5.8**). IL10 levels in all conditions were extremely low (data not shown). RtPCR was performed in parallel with these studies examining expression of IL12p35, IL12p40, and IL10 for correlation purposes (**Figure 5.9**, **Figure 5.10**, **Figure 5.11**, and **Figure 5.12**). In all conditions, IL12p40 cytokine levels (**Figure 5.5** and **Figure 5.6**) were significantly higher than IL12p70 levels (**Figure 5.7** and **Figure 5.8**). Cytokine expression levels were highest at 6 hours in most conditions that showed significant activation levels. This trend correlated to RT-PCR data for IL12p35 (**Figure 5.9** and **Figure 5.10**) and IL12p40 (**Figure 5.11** and **Figure 5.12**) levels. Interestingly, while there was no statistical difference between LPS activated IL12p40 levels at 6 hours, a statistically significant increase was seen as compared to OVA + CpG particles (both separately loaded as well as single loaded particle conditions) as time progressed. Similar to activation studies, there was no statistical difference in particle conditions containing siRNA vs. their OVA+CpG counterpart in separate loaded particles while there was a statistically significant difference in the dual vs. triple loaded particles at 48 hours (not at 24). IL12p70 levels were, however, significantly higher in LPS activated dendritic cells as compared to all conditions at all of the time points examined. Interestingly, particles loaded with CpG and OVA separately as compared to single particle loaded had significantly higher levels of IL12p70 expression at 6 hours but by 48

were no longer significantly higher. Dual and triple loaded single particle conditions behaved similarly. This may explain why there was little to no difference between dual and triple loaded particles in terms of DC activation markers. Il12p40 levels were not statistically different between separately and dual loaded particles in dual as compared to triple loaded conditions at 24 hours but at 48 hours, on dual loaded particles IL12p40 levels were statistically different in dual vs. triple particles.

5.4.2 *In vivo* tumor suppression

Tumor suppression was evaluated in both prophylactic and therapeutic models to assess the ability of our vaccine system to both prevent as well as treat mice infected with an aggressive melanoma tumor. Vaccine administration and injection schedule was based off of previous vaccination models (Li et al., 2011a). Tumor growth was monitored closely and mice were sacrificed based on tumor size as well as signs of ulceration. Statistical analysis was done using SPSS software. Long rank comparison was used to determine statistical significance. In our prophylactic model (**Figure 5.13** and **Figure 5.14**), all interventions performed statistically significantly better then PBS treated mice alone. Mice treated with dual and separately loaded microparticles, both containing CpG, performed statistically better than mice treated with only Ova loaded particles (without the addition of any other adjuvant). Here we also saw a statistically significant difference between separate and dual loaded particles, where the dual loaded particles performed better than the separately loaded particles. Further, there was no statistical difference between our dual loaded microparticles and our OVA IFA condition. Tumor growth (**Figure 5.12**) progressed rapidly in PBS control mice where tumors were measurable on the ninth day post-tumor cell injection. In mice receiving microparticles

that included CpG in their formulations, tumors were not observed until Day 22, after all control mice had already been sacrificed.

Differences in therapeutic models were similar (**Figure 5.15**, **Figure 5.16**, **Figure 5.17**, and **Figure 5.18**). In the first trial of these studies (**Figure 5.15** and **Figure 5.16**), all treatments produced a significant increase in mouse survival. For our second trial (**Figure 5.17** and **Figure 5.18**), particles loaded only with ovalbumin failed to induce a significant increase in survival from the untreated. Further, there failed to be any significant difference between the LPS, or CpG and OVA microparticle conditions. Whereas in our first trials, both conditions involving OVA antigen along with CpG provided a significant increase in mouse survival as compared to our positive LPS OVA control. In both studies, there existed no difference between the separately loaded microparticles and the dual loaded microparticle. One notable difference was the surprising delay in tumor growth in the second trial (**Figure 5.18**). This delay was observed in all conditions and was not a result of treatment. All mice purchased were the same weight (18-20 grams) and were allowed to acclimate to their environment the same amount of time (1 week). Again, when comparing the overall growth curves, you see that in the PBS negative control condition, once the tumors began to grow, their growth increased very quickly. In all other treatment cases, tumor growth was greatly delayed; this was especially seen in our second trial run.

5.5 DISCUSSION:

Usage of PLGA for the delivery of vaccine antigens and adjuvants is a widely researched field (Milacic et al., 2012) where the administration of PLGA with adsorbed or encapsulated antigens has been shown to improve responses in mammals as compared

to soluble antigen alone by improving uptake and processing of the antigen. PLGA microparticles can be effectively taken up by immature dendritic cells and does not result in maturation of DCs nor does it affect any ultimate functionality of DCs (Waeckerle-Men et al., 2004). Here, we took PLGA microparticles, imparted a cationic charge to the surface using PEI, a polymer that has been used as an effective non-viral transfection reagent, and used that cationic charge to electrostatically attach proteins and immunomodulatory molecules onto the surface.

Our first concern in our particulate system was the ability to induce maturation within our dendritic cells *in vitro*. To do this, we investigated two different immune modulating molecules: CpG and siRNA. Where our siRNA was acting not only as a potential immune activator (immune response to which is sequence specific (Marques and Williams, 2005) but also to actively silence production of a cytokine, IL10, in order to drive our T_H1 response. It has been previously shown that dendritic cells that are not activated (immature) can not only lead to anergy but actually lead to antigen-specific inhibition of preexisting effector T cell function by inducing IL10 production (Dhodapkar et al., 2001). Further, Lutz and Schuler suggest that the existence of a "semi-mature" state of DCs that produce CD4+IL10+Treg cells *in vivo* may induce tolerance (Lutz and Schuler, 2002). So here, we hypothesized that the inclusion of siRNA for IL10 would vastly improve our functionality of our microparticle system. While we did observe an increase in activation resulting from inclusion of the siRNA, overall the benefits of inclusion of the siRNA in our in vitro analysis was minor.

Because activation of the DCs was of the utmost importance, and because siRNA loaded particles did not induce high amounts of activation (**Chapter 4**) we also investigated the usage of CpG as an adjuvant, which has been extensively investigated for protein based vaccines (Davis et al., 1998; Diwan et al., 2002; Malyala et al., 2008; Xie,

Hang et al., 2005) and, further, has been investigated in human trials (Halperin et al., 2003). While most previously published work focused on encapsulation of CpG, we decided to use our PEI functionalized particles to surface load the CpG ensuring immediate availability for activation of TLR9 receptors. We saw a considerable increase in activation with our CpG being delivered via our particulate based system, similar to what has been observed in other studies (Singh et al., 2001; Xie, Hang et al., 2005). When used in combination with a weak antigen, p55gag, Singh et al. showed that using antigen loaded cationic PLGA microparticles delivered in combination with CpG loaded PLGA particles could elicit a significant CTL response (Singh et al., 2001). Delivering CpG loaded PLGA microparticles along with a protein for an anthrax vaccine resulted in a stronger and faster response then AVA alone or AVA in combination with soluble CpG (Xie, Hang et al., 2005). Here we looked to take these studies a step further, combining the usage of surface loaded siRNA specific to IL10, CpG and our model protein (OVA).

In all conditions investigated, activation was increased when immunostimulatory molecules were delivered on separate particles. While it may have been assumed that this increase in activation was a result of a larger number of microparticles, non-loaded PEI-PLGA microparticles did not induce a significant amount of DC maturation markers (as compared to non-activated DCs). We furthered our *in vitro* analysis by looking at cytokine secretion as well as gene expression. When we investigated gene expression as well as cytokine secretion we saw that, generally speaking, a lack of difference between the dual vs. triple loaded formulations when comparing their single vs. multiple loaded microparticle formulations. For this reason, along with cost considerations for ultimate end applications, we decided to move forward with our investigations using our dual loaded systems.

Our in vivo studies were modeled after similar studies completed by other labs using other OVA based particulate delivery systems (Li et al., 2011a). While our studies are preliminary, their implications are very interesting. For our prophylactic model, we observed that tumor growth in the PBS control group accelerated quickly and was followed closely by our particles containing OVA without any CpG present. Contrary to the general trend observed in our in vitro data, particles loaded with both CpG and OVA performed statistically better than the separately loaded particles in a prophylactic model. This is contrary to observations seen by other groups (Malyala et al., 2008). In our therapeutic model, this same significance wasn't seen, though the mice receiving dual loaded particles did survive longer. A second trial indicated the same trend, a lack of statistical difference between dual and separately delivered antigen and adjuvant. These results indicate that we have developed a protein based vaccine delivery system capable of having significant *in vivo* effects on tumor growth. While these studies are promising, further work needs to be completed to fully establish, mechanistically, that the vaccine is creating a protein-specific response. Plans for future studies will be further discussed in the next chapter (**Chapter 6)**

Table 5.1: Basic characterization of PEI-PLGA microparticles for protein and immunomodulatory molecule delivery

			Loading Efficiency
Size		935 ±10 nm	
Zeta Potential	before PEI	-6.29 ±1.2 mV	
	after PEI	+ 32.77 ±0.7 mV	
Single Loading (µg/mg PLGA)	OVA	10.9±0.03	90%
	CpG ODNs	11.72±0.03	97%
	siRNA	9.8±0.1	82%
Dual Loading (µg/mg PLGA)	OVA + CpG	11.4±0.09 11.9±.01	95% 99%
Triple Loading (µg/mg PLGA)	OVA + siRNA + CpG	12.744±0. 10.3±0.5 12.14±1.13	85% 69% 81%

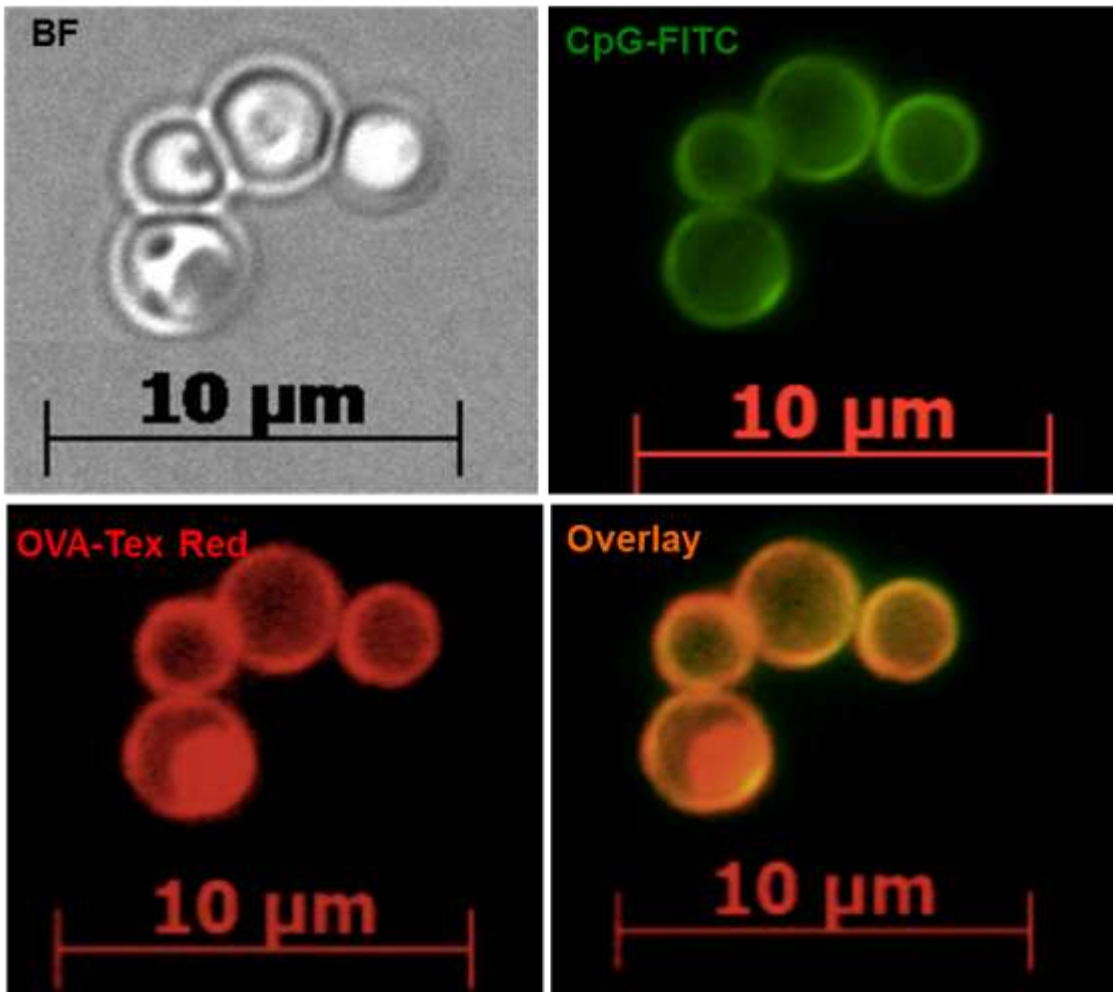

Figure 5.1: Confocal microscopy image of dual loaded microparticles

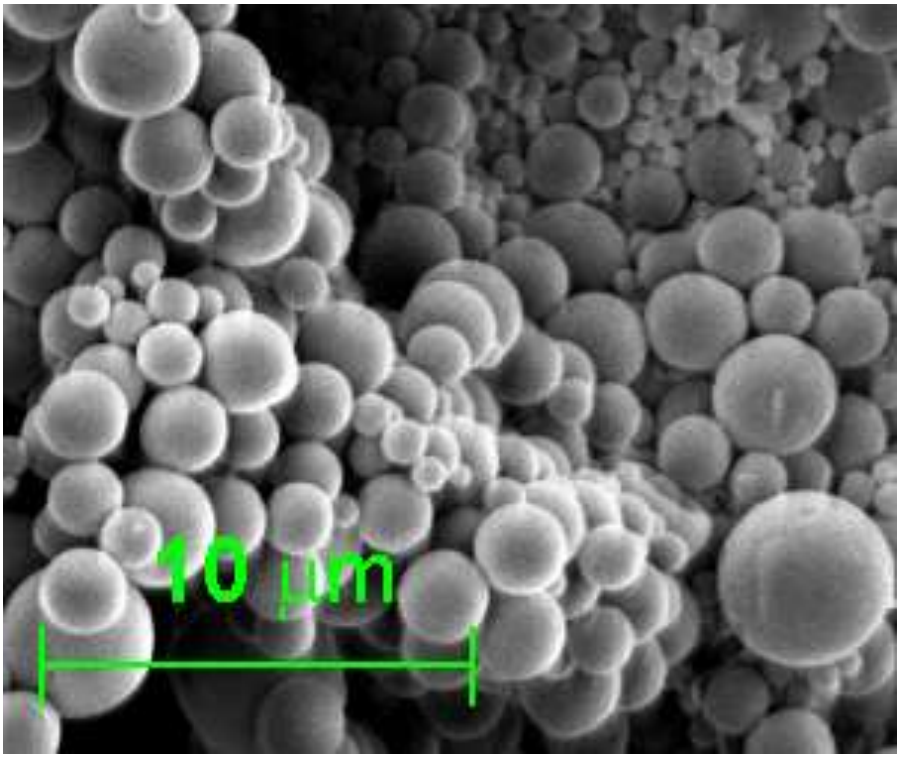

Figure 5.2 Scanning electron microscopy image of our PEI-PLGA microparticles: SEM images were taken following the same protocol as discussed in **Chapter 3**. Here we observe a polydispersed population of particles with a smooth surface.

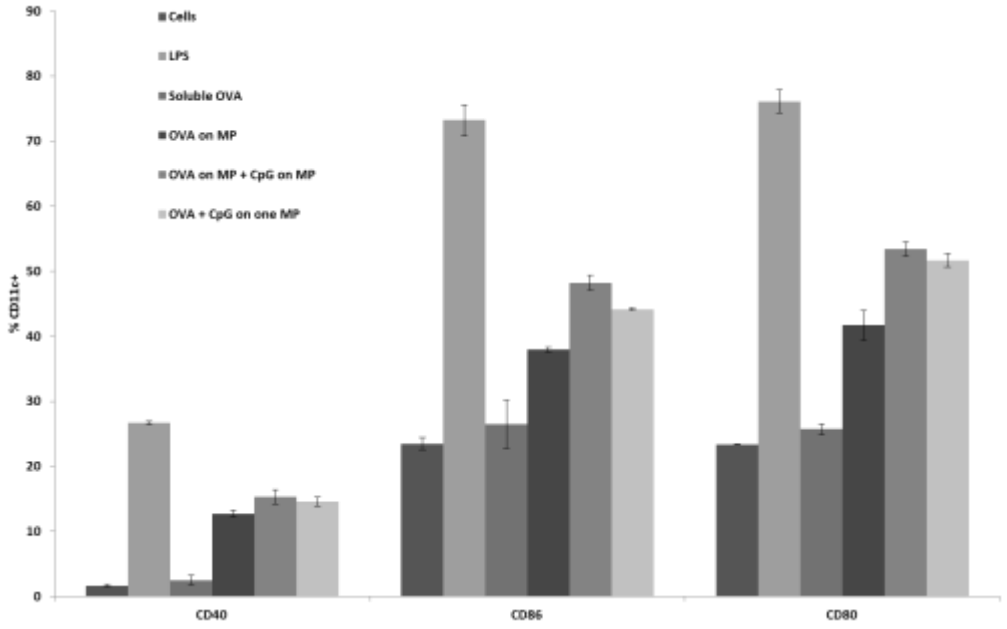

Figure 5.3 Dendritic cell surface activation marker expression in response to dual loaded microparticles: Dendtric cell activation was characterized by examining cell surface expression of primary dendritic cells differentiated from bone marrow of Balb/c mice for 6-7 days via flow cytometry. For each experimental condition, each well received 1 μg of ovalbumin and cells were activated for 48 hours. Data is representative of multiple independent experiments. For all conditions appropriate soluble controls were performed in addition to particulate conditions. Data shown is surface marker expression of only live, CD11c+ dendritic cells (representing approximately 80% of the total live cell population).

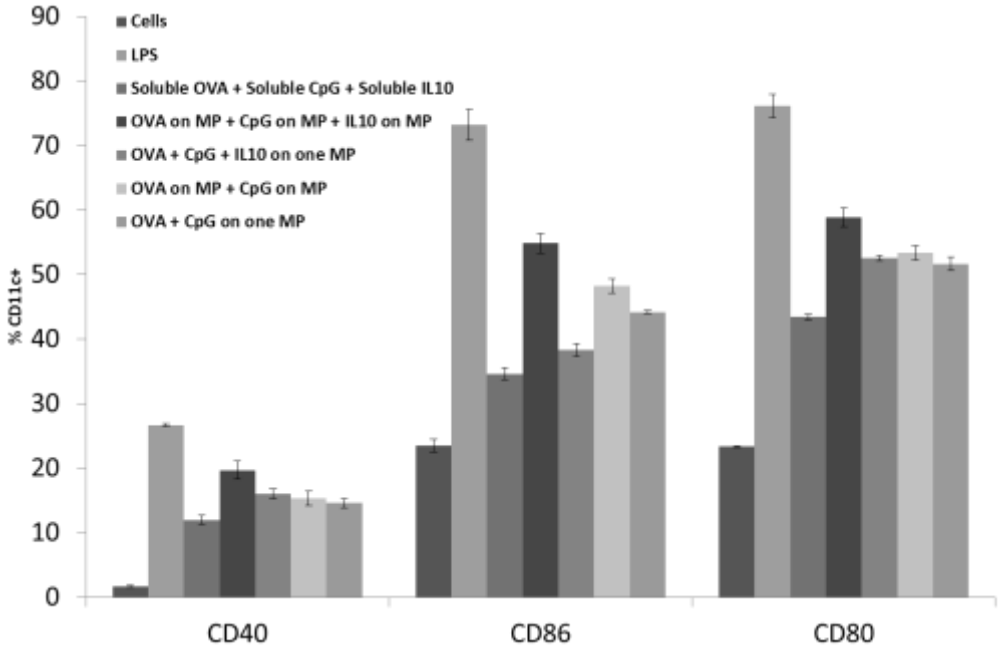

Figure 5.4 Dendritic cell surface marker expression in response to activation using triple loaded microparticles: Dendtric cell activation was characterized by examining cell surface expression of primary dendritic cells differentiated from bone marrow of Balb/c mice for 6-7 days. For each experimental condition, each well received 1 μg of ovalbumin and cells were activated for 48 hours. Data is representative of multiple independent experiments. For all conditions appropriate soluble controls were performed in addition to particulate conditions. Data shown is surface marker expression of only live, CD11c+ dendritic cells (representing approximately 80% of the total live cell population).

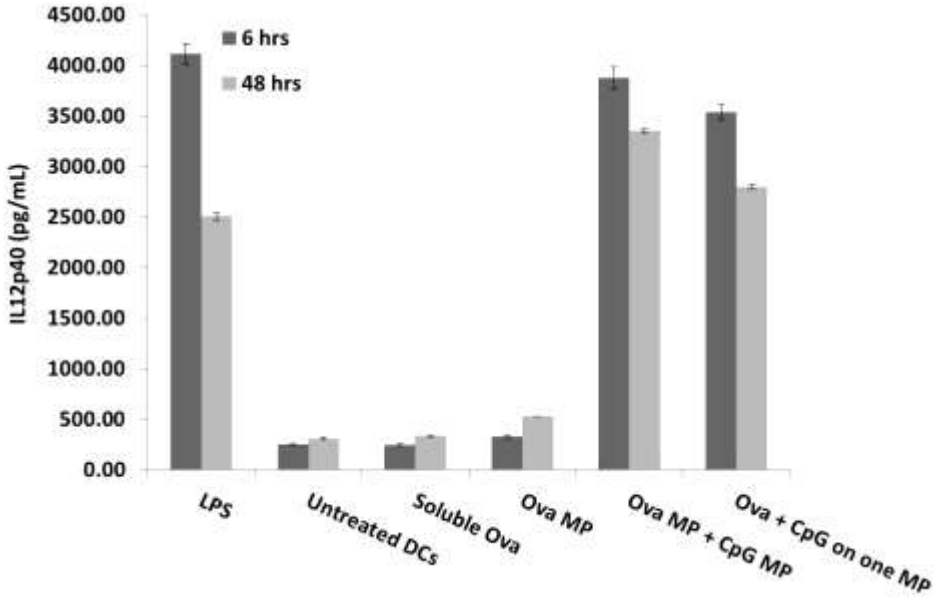

Figure 5.5 IL12p40 secretion from dendritic cells activated with dual loaded microparticles: Dendritic cells were isolated and cultured in the same manner as with previous activation studies. At 6, 24, and 48 hours after addition of particulate formulations and the same dosages used in the activation studies, media was removed and cytokine secretion was determined using a Ready-Set-Go!® Elisa kit.

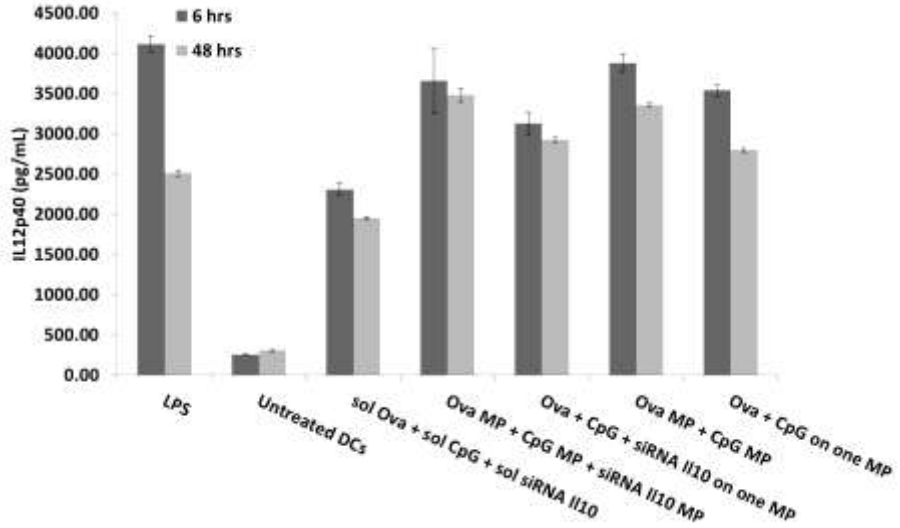

Figure 5.6 IL12p40 secretion from dendritic cells activated with triple loaded microparticles: Dendritic cells were isolated and cultured in the same manner as with previous activation studies. At 6, 24, and 48 hours after addition of particulate formulations, media was removed and cytokine secretion was determined using a Ready-Set-Go!® Elisa kit.

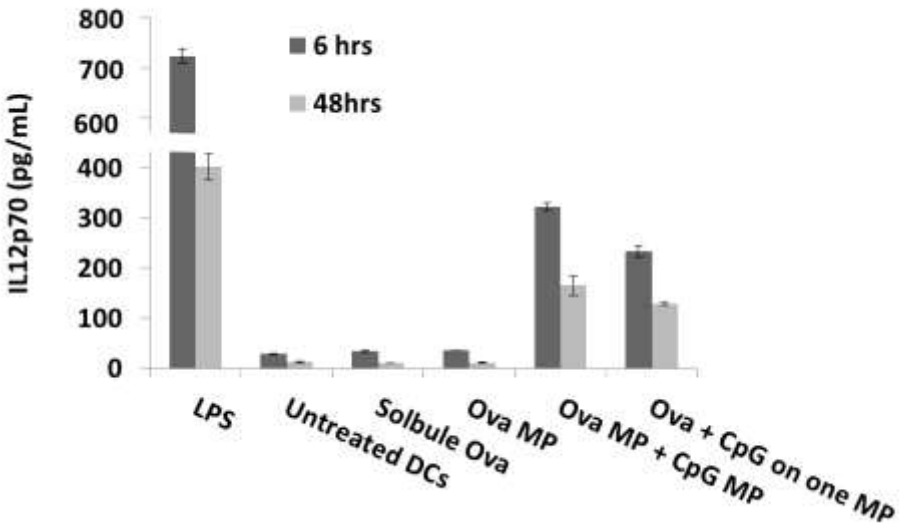

Figure 5.7 IL12p70 secretion by dendritic cells activated using dual loaded microparticles: Dendritic cells were isolated and cultured in the same manner as with previous activation studies. At 6, 24, and 48 hours after addition of particulate formulations, media was removed and cytokine secretion was determined using a Ready-Set-Go!® Elisa kit.

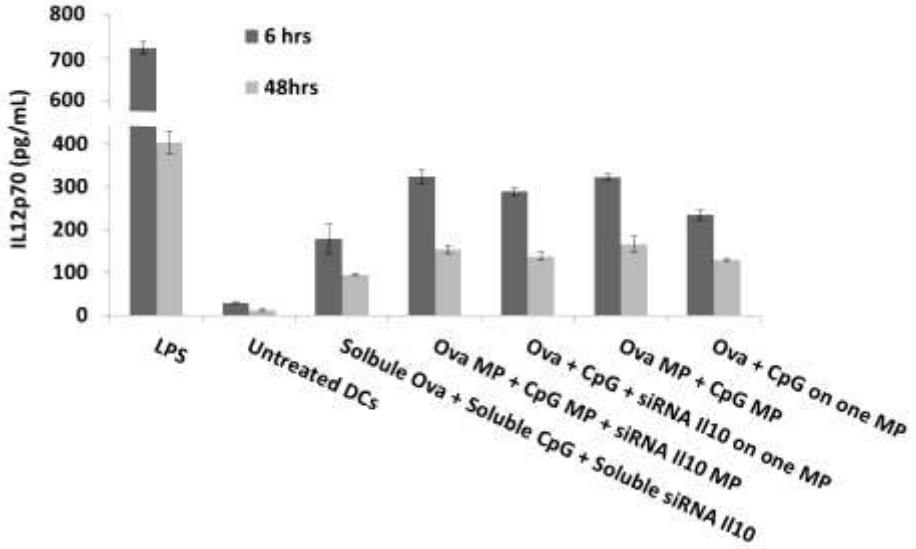

Figure 5.8 IL12p70 secretion by dendritic cells activated with triple loaded microparticle formulations: Dendritic cells were isolated and cultured in the same manner as with previous activation studies. At 6, 24, and 48 hours after addition of particulate formulations, media was removed and cytokine secretion was determined using a Ready-Set-Go!® Elisa kit.

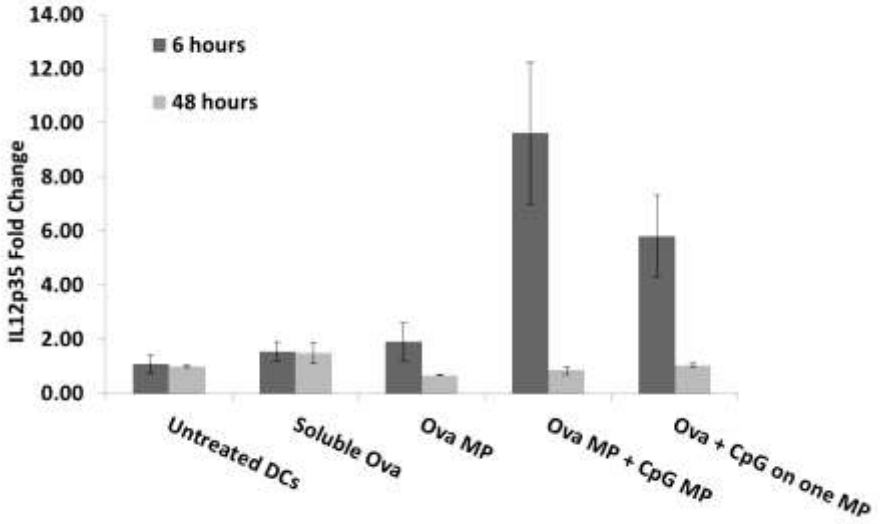

Figure 5.9 Change in gene expression of IL12p35 in response to dual loaded microparticle formulations: Total RNA was extracted from BMDCs and cDNA was synthesized from the RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen), and real time RT-PCR was performed using RT² SYBR® green qPCR mastermix (Qiagen). Relative gene expression level for the target gene were calculated following ΔΔCT method using the formula: Target gene expression of sample = 2(−ΔΔCT), where ΔΔCT = [CT (target gene) − CT (reference gene)] of treatment − [CT (target gene) − CT (reference gene)] of control and the threshold cycle (CT) is the PCR cycle at which first signal of reporter fluorescence above a baseline signal is detected.

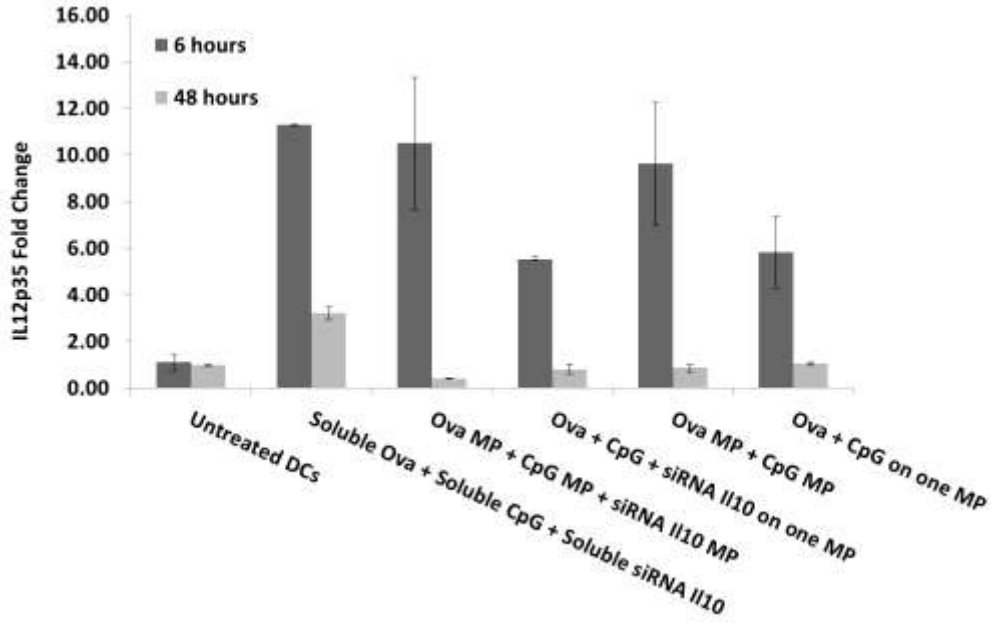

Figure 5.10 IL12p35 gene expression in triple loaded microparticle activated dendritic cells: Total RNA was extracted from BMDCs and cDNA was synthesized from the RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen), and real time RT-PCR was performed using RT² SYBR® green qPCR mastermix (Qiagen). Relative gene expression level for the target gene were calculated following ΔΔCT method using the formula: Target gene expression of sample = 2(−ΔΔCT), where ΔΔCT = [CT (target gene) − CT (reference gene)] of treatment − [CT (target gene) − CT (reference gene)] of control and the threshold cycle (CT) is the PCR cycle at which first signal of reporter fluorescence above a baseline signal is detected.

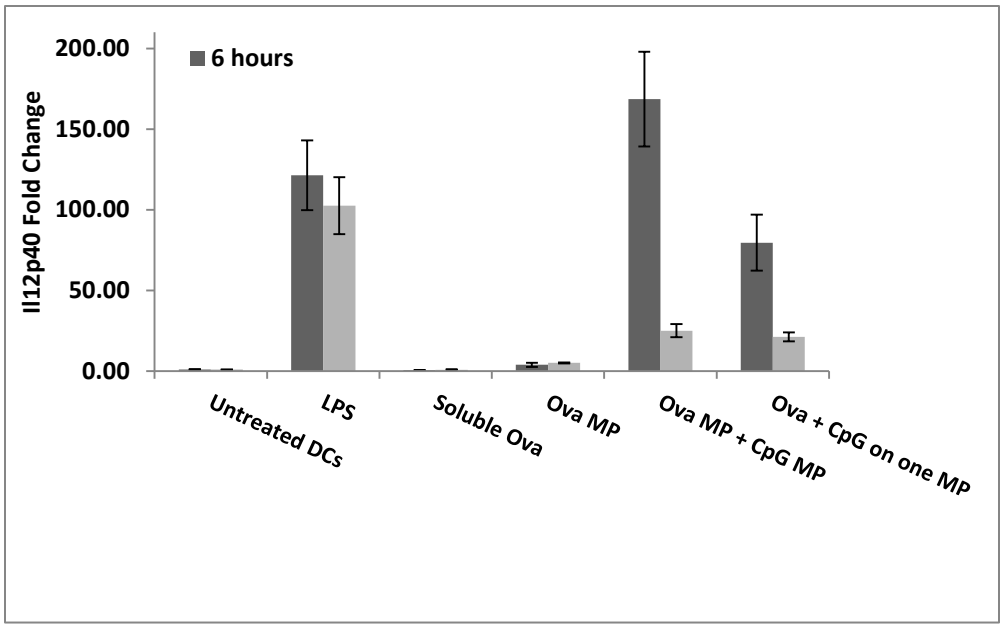

Figure 5.11 IL12p40 gene expression in dendritic cells activated by dual loaded microparticles: Total RNA was extracted from BMDCs and cDNA was synthesized from the RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen), and real time RT-PCR was performed using RT² SYBR® green qPCR mastermix (Qiagen). Relative gene expression level for the target gene were calculated following ΔΔCT method using the formula: Target gene expression of sample = 2(−ΔΔCT), where ΔΔCT = [CT (target gene) − CT (reference gene)] of treatment − [CT (target gene) − CT (reference gene)] of control and the threshold cycle (CT) is the PCR cycle at which first signal of reporter fluorescence above a baseline signal is detected.

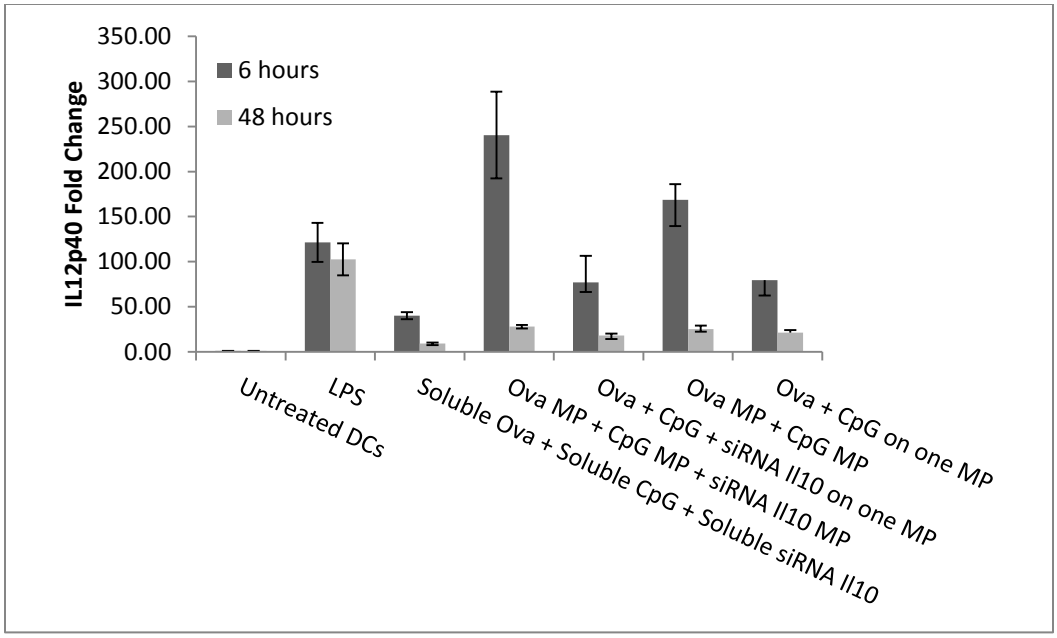

Figure 5.12 Il12p40 gene expression in triple loaded microparticle formula activated dendritic cells: Total RNA was extracted from BMDCs and cDNA was synthesized from the RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen), and real time RT-PCR was performed using RT² SYBR® green qPCR mastermix (Qiagen). Relative gene expression level for the target gene were calculated following ΔΔCT method using the formula: Target gene expression of sample = 2(−ΔΔCT), where ΔΔCT = [CT (target gene) − CT (reference gene)] of treatment − [CT (target gene) − CT (reference gene)] of control and the threshold cycle (CT) is the PCR cycle at which first signal of reporter fluorescence above a baseline signal is detected.

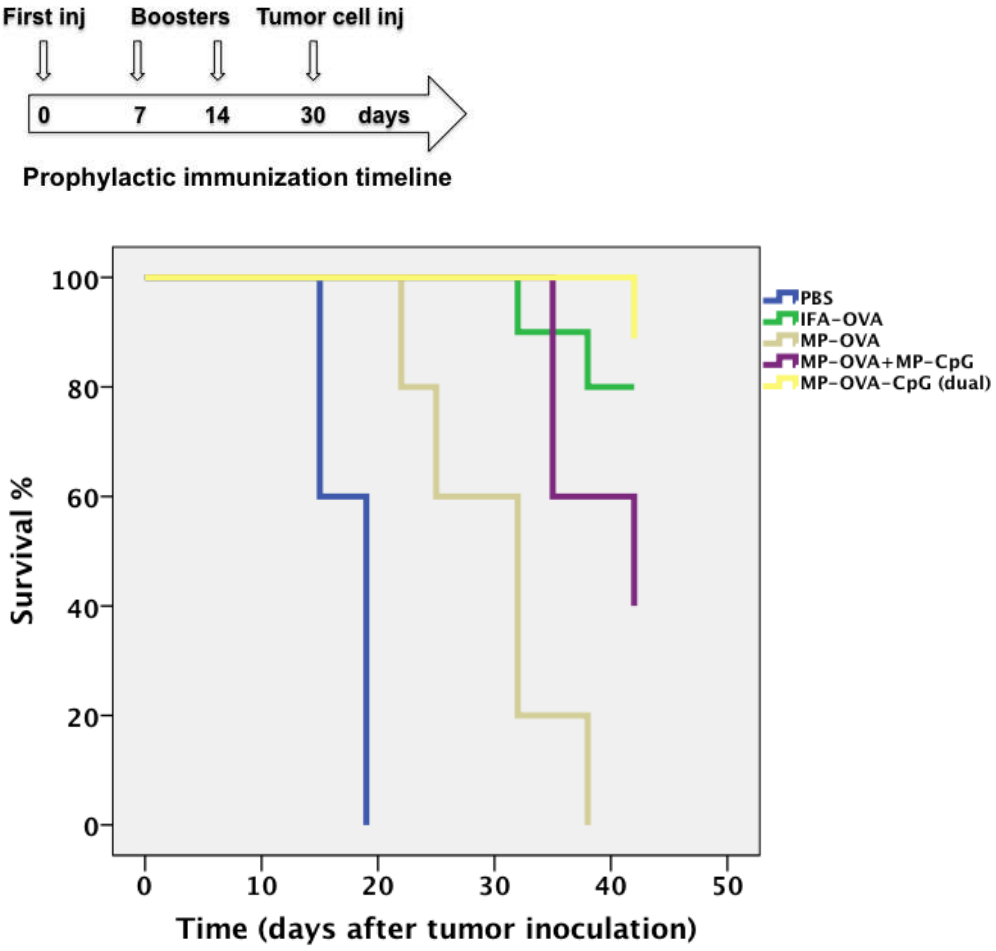

Figure 5.13 B16 OVA expressing melanoma prophylactic model survival curve and injection schedule: Here we show a schematic outline of our injection schedule. Below it is our survival curve of mice in prophalactic model. Mice were sacrificed when tumor volume reached over 400 mm^3 calculated using a modified ellipsoidal formula following formula: tumor volume= ½ (length*$width^2$) (Euhus et al., 1986; Jensen et al., 2008; Tomayko and Reynolds, 1989).

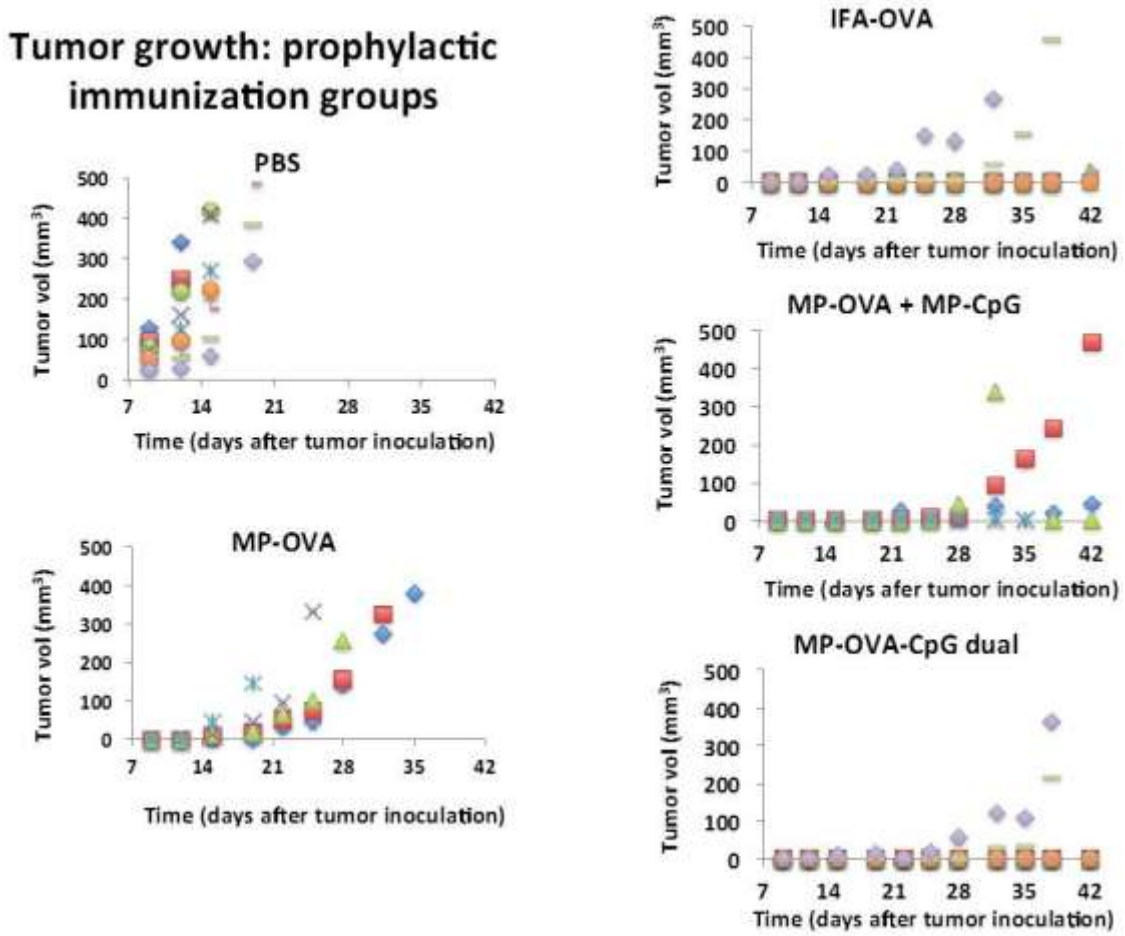

Figure 5.14 Prophylactic *in vivo* model tumor growth curves: Tumor growth was monitored frequently and volume was calculated using the following equation: tumor volume= ½ (length*width^2) (Euhus et al., 1986; Jensen et al., 2008; Tomayko and Reynolds, 1989).

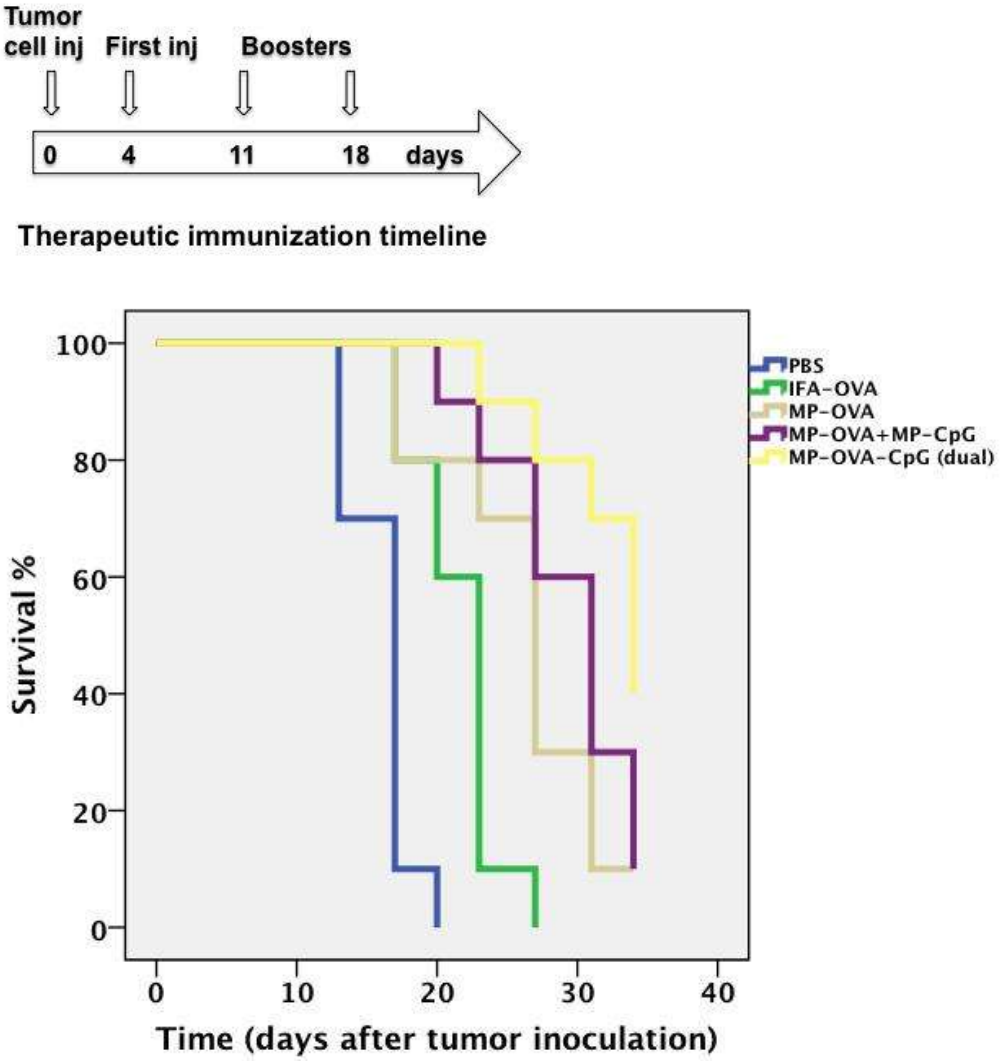

Figure 5.15 Initial trail for therapeutic vaccination in B16 OVA expressing melanoma: Here we show a schematic outline of our injection schedule. Below it is our survival curve of mice in our first trial of our therapeutic treatment study. Mice were sacrificed when tumor volume reached over 400 mm3 calculated using a modified ellipsoidal formula following formula: tumor volume= ½ (length*width2) (Euhus et al., 1986; Jensen et al., 2008; Tomayko and Reynolds, 1989).

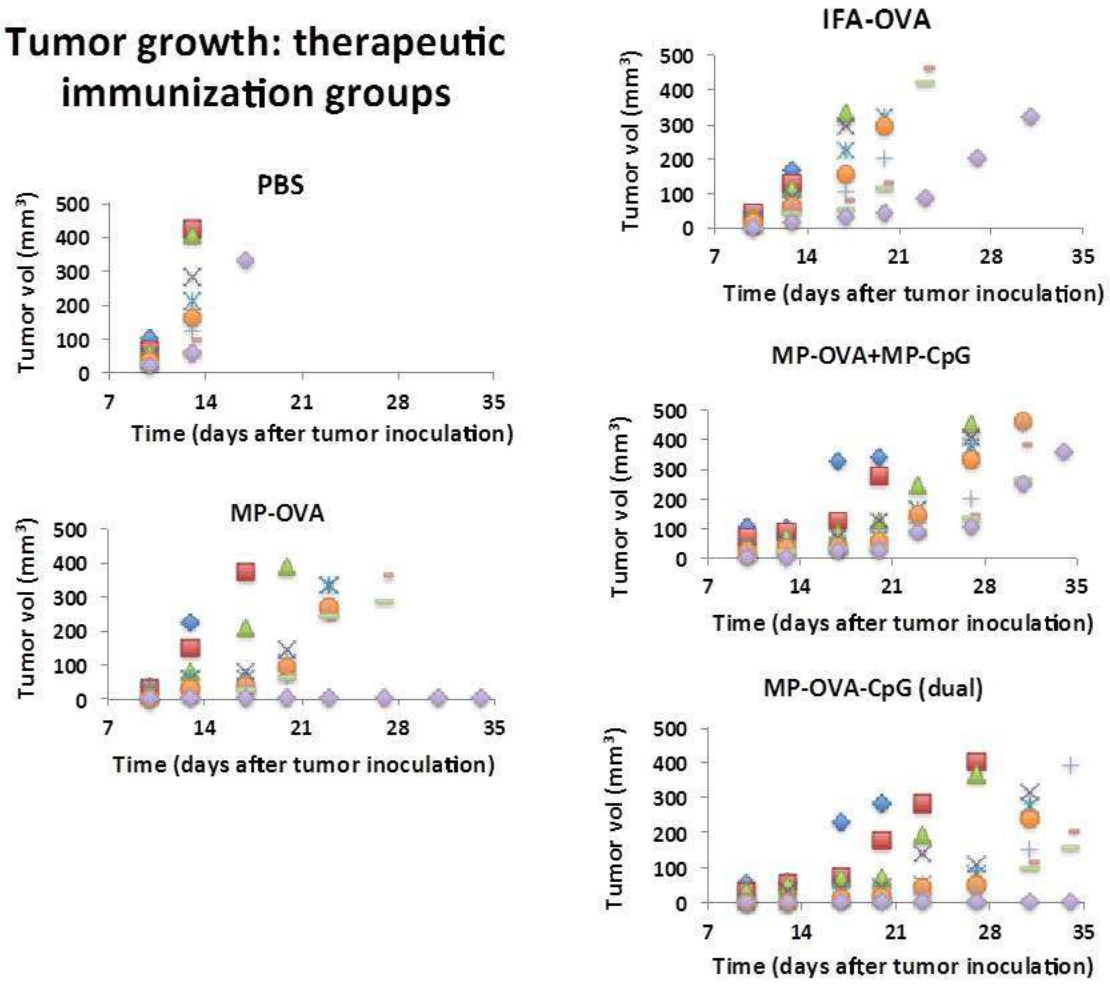

Figure 5.16 Tumor volume growth in first trial of therapeutic model for ovalbumin expressing melanoma: Tumor growth was monitored frequently and volume was calculated using the following equation: tumor volume= ½ (length*width^2) (Euhus et al., 1986; Jensen et al., 2008; Tomayko and Reynolds, 1989).

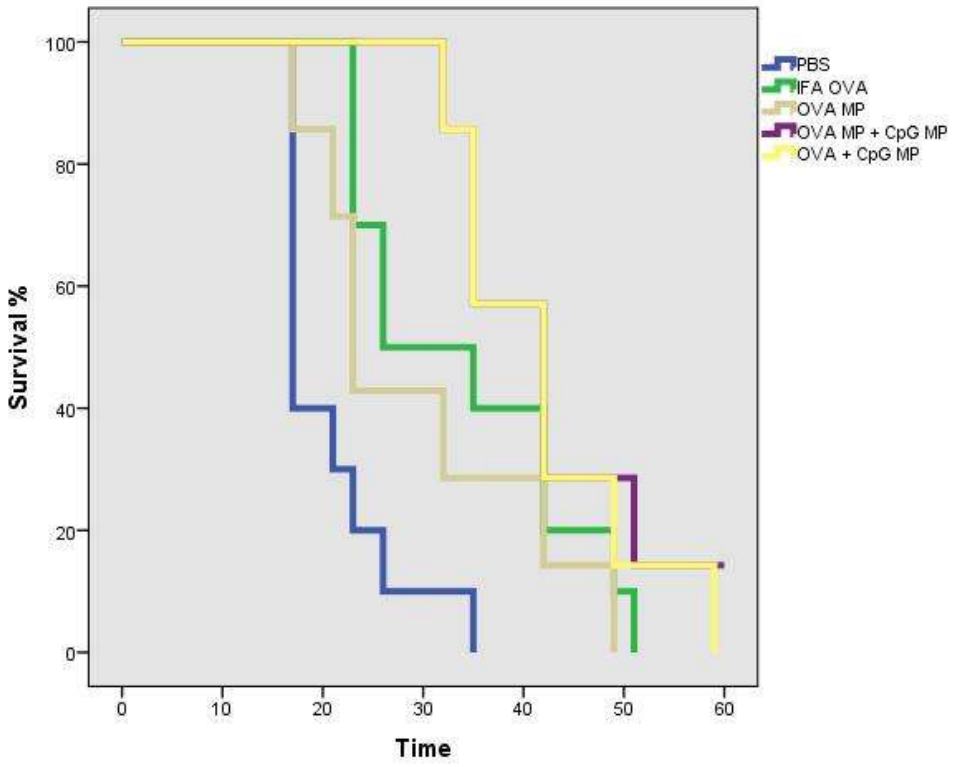

Figure 5.17 Second trial of therapeutic vaccine use for B16 melanoma model: Following the same injection schedule as before, here we show our survival curve of mice in our first trial of our therapeutic treatment study. Mice were sacrificed when tumor volume reached over 400 mm^3 calculated using a modified ellipsoidal formula following formula: tumor volume= ½ (length*$width^2$) (Euhus et al., 1986; Jensen et al., 2008; Tomayko and Reynolds, 1989).

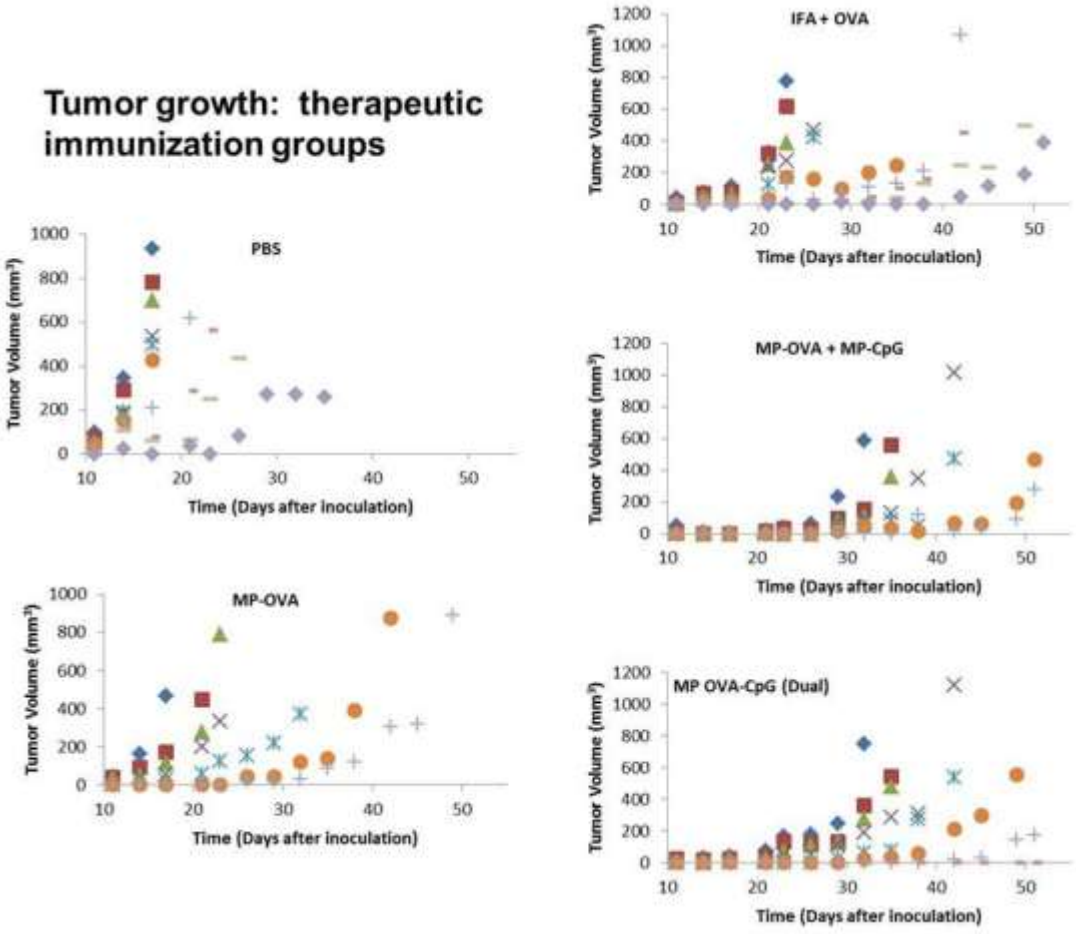

Figure 5.18 Tumor volume growth in second trial in therapeutic treatment model for B16 melanoma tumor: Tumor growth was monitored frequently and volume was calculated using the following equation: tumor volume= ½ (length*width^2) (Euhus et al., 1986; Jensen et al., 2008; Tomayko and Reynolds, 1989).

5.6 REFERENCES

Davis, H.L., Weeranta, R., Waldschmidt, T.J., Tygrett, L., Schorr, J., and Krieg, A.M. (1998). CpG DNA Is a Potent Enhancer of Specific Immunity in Mice Immunized with Recombinant Hepatitis B Surface Antigen. J. Immunol. 160, 870–876.

Dhodapkar, M.V., Steinman, R.M., Krasovsky, J., Munz, C., and Bhardwaj, N. (2001). Antigen-Specific Inhibition of Effector T Cell Function in Humans after Injection of Immature Dendritic Cells. J. Exp. Med. 193, 233–238.

Diwan, M., Tafaghodi, M., and Samuel, J. (2002). Enhancement of immune responses by co-delivery of a CpG oligodeoxynucleotide and tetanus toxoid in biodegradable nanospheres. J. Controlled Release 85, 247–262.

Ehlers, S., and Bulfone-Paus, S. (2004). Shaping Adaptive Immunity against Pathogens: The Contribution of Innate Immune Responses. In Novel Vaccination Strategies, S.H.E. Kaufmann, ed. (Wiley-VCH Verlag GmbH & Co. KGaA), pp. 17–50.

Euhus, D.M., Hudd, C., Laregina, M.C., and Johnson, F.E. (1986). Tumor measurement in the nude mouse. J. Surg. Oncol. 31, 229–234.

Halperin, S.A., Van Nest, G., Smith, B., Abtahi, S., Whiley, H., and Eiden, J.J. (2003). A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. Vaccine 21, 2461–2467.

Hermans, I.F., Silk, J.D., Gileadi, U., Masri, S.H., Shepherd, D., Farrand, K.J., Salio, M., and Cerundolo, V. (2007). Dendritic Cell Function Can Be Modulated through Cooperative Actions of TLR Ligands and Invariant NKT Cells. J. Immunol. 178, 2721–2729.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 176, 1693–1702.

Jensen, M.M., Jørgensen, J.T., Binderup, T., and Kjær, A. (2008). Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper. Bmc Med. Imaging 8, 16.

Kasturi, S.P., Sachaphibulkij, K., and Roy, K. (2005). Covalent conjugation of polyethyleneimine on biodegradable microparticles for delivery of plasmid DNA vaccines. Biomaterials 26, 6375–6385.

Li, X., Sloat, B.R., Yanasarn, N., and Cui, Z. (2011). Relationship between the size of nanoparticles and their adjuvant activity: Data from a study with an improved experimental design. Eur. J. Pharm. Biopharm. 78, 107–116.

Lutz, M.B., and Schuler, G. (2002). Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol. 23, 445–449.

Malyala, P., Chesko, J., Ugozzoli, M., Goodsell, A., Zhou, F., Vajdy, M., O'Hagan, D.T., and Singh, M. (2008). The potency of the adjuvant, CpG oligos, is enhanced by encapsulation in PLG microparticles. J. Pharm. Sci. 97, 1155–1164.

Marques, J.T., and Williams, B.R.G. (2005). Activation of the mammalian immune system by siRNAs. Nat. Biotechnol. 23, 1399–1405.

Milacic, V., Bailey, B.A., O'Hagan, D., and Schwendeman, S.P. (2012). Injectable PLGA Systems for Delivery of Vaccine Antigens. In Long Acting Injections and Implants, J.C. Wright, and D.J. Burgess, eds. (Springer US), pp. 429–458.

Pai Kasturi, S., Qin, H., Thomson, K.S., El-Bereir, S., Cha, S.-C., Neelapu, S., Kwak, L.W., and Roy, K. (2006). Prophylactic anti-tumor effects in a B cell lymphoma model with DNA vaccines delivered on polyethylenimine (PEI) functionalized PLGA microparticles. J. Control. Release Off. J. Control. Release Soc. 113, 261–270.

Perrie, Y., Mohammed, A.R., Kirby, D.J., McNeil, S.E., and Bramwell, V.W. (2008). Vaccine adjuvant systems: Enhancing the efficacy of sub-unit protein antigens. Int. J. Pharm. 364, 272–280.

Salvador, A., Igartua, M., Hernández, R.M., and Pedraz, J.L. (2012). Combination of immune stimulating adjuvants with poly(lactide-co-glycolide) microspheres enhances the immune response of vaccines. Vaccine 30, 589–596.

Singh, A., Nie, H., Ghosn, B., Qin, H., Kwak, L.W., and Roy, K. (2008). Efficient Modulation of T-cell Response by Dual-mode, Single-carrier Delivery of Cytokine-targeted siRNA and DNA Vaccine to Antigen-presenting Cells. Mol. Ther. 16, 2011–2021.

Singh, A., Suri, S., and Roy, K. (2009). In-situ crosslinking hydrogels for combinatorial delivery of chemokines and siRNA-DNA carrying microparticles to dendritic cells. Biomaterials 30, 5187.

Singh, M., Ott, G., Kazzaz, J., Ugozzoli, M., Briones, M., Donnelly, J., and O'Hagan, D.T. (2001). Cationic Microparticles Are an Effective Delivery System for Immune Stimulatory CpG DNA. Pharm. Res. 18, 1476–1479.

Thompson, C.B. (1995). Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation? Cell 81, 979–982.

Tomayko, M.M., and Reynolds, C.P. (1989). Determination of subcutaneous tumor size in athymic (nude) mice. Cancer Chemother. Pharmacol. 24, 148–154.

Waeckerle-Men, Y., Scandella, E., Uetz-von Allmen, E., Ludewig, B., Gillessen, S., Merkle, H.P., Gander, B., and Groettrup, M. (2004). Phenotype and functional

analysis of human monocyte-derived dendritic cells loaded with biodegradable poly(lactide-co-glycolide) microspheres for immunotherapy. J. Immunol. Methods 287, 109–124.

Xie, Hang, Gursel, Ihsan, Ivins, Bruce E, Singh, Monmohan, O'Hagan, Derek T, Ulmer, J.B., and Klinman, D.M. (2005). CpG Oligodeoxynucleotides Adsorbed onto Polylactide-Co-Glycolide Microparticles Improve the Immunogenicity and Protective Activity of the Licensed Anthrax Vaccine. Infect. Immun. 73, 828–833.

CHAPTER 6

Conclusion and Future Directions

6.1 SUMMARY:

We have demonstrated the applicability of using charged microparticle systems for protein antigen delivery along with adjuvant immune modulating molecules for the potential usage as a platform vaccine system. The cationic protein loaded particles have demonstrated, when delivered in combination with adjuvant molecules, that they are able to illicit dendritic cell activation *in vitro* as well as offer some protective as well as therapeutic effects *in vivo* in our ovalbumin expressing melanoma model. For our anionic protein delivery system, we have completed a set of preliminary studies indicating that our dry functionalization method (plasma surface modification) can enhance the anionic property of the surface of PLGA microparticles and can offer an effective substrate for the electrostatic attachment of cationic proteins. Our results indicate two promising systems for the delivery of protein adjuvants.

6.1.1 CONCLUSIONS ON OUR PLASMA MODIFIED PLGA MICROPARTICLE BASED ANTIGEN DELIVERY SYSTEM

Plasma modification techniques have been widely used to develop a more bioactive or biocompatible surface in many different applications (Alves et al., 2007, 2008; Armentano et al., 2009; Bélard et al., 2013; Desmet et al., 2009; Hasirci et al., 2010). Here we demonstrated usage in a microparticle based system where the particles were modified by coating on a glass cover slide and being exposed to plasma. In the literature, this type of application of plasma modification has yet to be reported (most work focuses on either modification of the polymers themselves or of polymer based films). We showed, that for our purposes, we were able to impart a change in surface

functionality as indicated by a clear shift in zeta potential of our particles. We investigated a number of different plasma types (atmospheric and low pressure), gases (helium and oxygen), as well as other modulatory factors according to plasma source in order to optimize our system. While experiments were in process, we began to observe sudden shifts in zeta potential from our atmospheric glow discharge system. Specifically, we were observing high run-to-run variability in the observed zeta potentials. The differences noticed could most likely be a result of an inherent flaw in the system discussed by Shin and Raja where shorter plasma modification times result in a difference in voltage-current waveforms at the start of each new run (Shin and Raja, 2007). While this wouldn't matter for longer plasma exposure, it does matter for our purposes since we need to minimize the ablative effects of longer exposure times on our microparticles (Wan et al., 2004).

We next began investigating the usage of low-pressure based systems. The observed results were highly consistent (as compared to the atmospheric system). Further, we were able to load lysozyme at very high efficiencies (over 98% at a 1.2 wt% loading). SEM images revealed what appeared to be slightly rougher surfaces of the plasma modified particles. We next investigated surface aging (Morent et al., 2007; Vesel and Mozetic, 2012). In our particles, we observed significant decreases in zeta potential of our particles in short periods of time. Similarly, Morent et al. observed drastic changes in surface properties within the first 48 hours of storage of polypropylene and polyethylene terephthalate films. Their studies suggested that the choice of gas during plasma modification played a key role in the effects of aging. Specifically, they suggested that the more cross-linked the surface of the polymer film was, the more likely able it was to resist the effects of aging (Morent et al., 2007). While this could provide stability of the charge on the surface of the particles, it would limit the diffusion of

material out of the particles should we move forward with encapsulation of another agent within the particles. We have primarily focused on creating a balance between the charge of the particle while limiting the exposure time of the particles to plasma, not only to limit the degradative effects of plasma, but also to keep open the option of encapsulation of a bioactive agent within the particles (Eisenbrey et al., 2009; Holy et al., 2001).

6.1.2 CONCLUSIONS ON OUR PEI MODIFIED PLGA MICROPARTICLE BASED ANTIGEN DELIVERY SYSTEM

We have shown that by covalently conjugating branched polyethyenemine we can significantly increase the zeta potential of PLGA microparticles. Using this cationic charge, we can alter loading conditions (we did this by altering the type of buffer and their respective pHs) to drive electrostatic interactions and increase the ability of the particles to adsorb proteins (Chapter 4). With the inclusion of immunomodulatory molecules, we can increase the activation of primary dendritic cells. Different combinations of antigenic protein and immunomodulatory molecules (CpG and siRNA for IL10) resulted in different expression profiles of primary DC activation markers as well as secretion of cytokines and gene expression.

We also investigated the difference between delivering our protein antigen in combination with our immunomodulatory molecules on the same particle vs. separate particles (Chapter 5). Generally speaking, we found that triple loaded particles (CpG, siRNA, and OVA) as compared to our dual loaded particles (CpG and OVA) did not increase *in vitro* DC activation enough to warrant the cost of *in vivo* investigation. Therefore, for our purposes, we continued our investigations with only our dual loaded particles and their single particle controls. Our preliminary studies indicate that as a prophylactic vaccine, our particles can provide a significant amount of protection and that

the dual loaded particles produce the highest statistically significant increase in protection. Our therapeutic trial results were not as definitive. Here we observed that the dual vs. separate particles did not perform statistically different *in vivo* (nor did either particulate system perform better than our positive control, IFA and OVA).

As suggested by Kasturi et al. (Kasturi et al., 2011) the ability to deliver antigens and adjuvants on separate particles offers an interesting new platform for coupling a generic adjuvant particle with another particle containing an antigen from a different pathogen. This separately functional adjuvant loaded particle could act, not only in an antigen sparing way but could also, ultimately reduce the number of vaccinations a person needs to receive (i.e. it may be possible to receive a vaccination against multiple antigens at the same time, assuming the type of immune response desired is the same).

6.1.3 RECOMMENDATIONS FOR FUTURE WORK

Currently, we have investigated usage of two differently charged particle systems based upon using PLGA microparticles and then altering their surface functionality to electrostatically attach protein antigens to their surfaces. We have focused exclusively on electrostatics to surface load molecules onto our particles in our investigations. If our anionic plasma modified particles behave similarly to our PEI-PLGA microparticles, an adjuvant may be necessary to elicit an immune response. As previously suggested in 6.1.1 there are a number of concerns that would need to be addressed with this. First and foremost we would need to establish that any encapsulated agent remains bioactive after plasma surface modification. Depending on the adjuvant/immunomodulatory molecule used this can be done easily in a number of ways. If we continue using an siRNA molecule, a GAPDH siRNA can be used as a model siRNA, extracted after exposure to

plasma, and a KDalert™ Assay can be performed. Similarly we could test the functionality of other active molecules on their effects on primary immune cells (i.e. compare non-plasma exposed molecules to exposed molecules). As suggested in section 6.1.2, if having our adjuvant on the same molecule vs. separate molecules does not affect ultimate functionality *in vivo* of the system, alternatively, it may be possible to use both our plasma modified particles (loaded with protein antigen) and our cationic particles (loaded with adjuvant) in combination with one another.

The aging of the surface of our plasma modified microparticles is also an issue that needs to be addressed. Obviously, this is a well-documented phenomenon, but attempts to prevent it have met with limited success. Most aging studies occur in less than ideal storage conditions (Li et al., 2003; Morent et al., 2007; Vesel, 2010). It has already been established that, at least in the case of helium plasma treatment, the surface actually reacts with the environment to gain its new functional groups. We have shown (Chapter 3) that lyophilization overnight does not change the zeta potential of our particles. Perhaps limiting the interaction of the microparticles with air would prevent some of this surface relaxation. Further, as with most reactions, temperature can play a very important kinematic role. Therefor there may be several different storage conditions we could evaluate to reduce the effects of aging. Further, we may stabilize the functional groups by actually loading our protein antigens onto the surface of the particles immediately and storing them pre-loaded. This would reduce some of the functionality of the vaccine system in that they would have to be pre-loaded with antigen, would still be a satisfactory alternative if the particles are not stable in conditions that would be favorable for long-term storage.

Once these two topics have been investigated, we could begin studying the therapeutic effects of these particles with cationic proteins *in vivo* to elucidate their effectiveness in an appropriate model.

In vivo trials of our particulate formulations have only just begun. As mentioned previously, our prophylactic study has only been completed a single time, and thus, needs to be repeated to confirm our initial findings. Further, additional groups need to be investigated within our system. Specifically, we need to evaluate the effects of CpG microparticles alone on tumor protection. It has been demonstrated that CpG alone can have a therapeutic effect when delivered without a protein antigen (Heckelsmiller et al., 2002; Kigasawa et al., 2011). We therefore would need to both test the effects that CpG microparticles alone have on our system as well as investigate the specificity of the immune response that we have generated. One way to do this would be to perform an *in vivo* cytotoxic T-cell assay (Cui and Qiu, 2006; Li et al., 2011b). Briefly, this would involve vaccinating mice with our formulations and then introducing splenocytes from naïve mice that have been pulsed with OVA as well as labeled with CFSE. In this way it would be possible to determine if our results were specific to cells producing the protein antigen or if it was a direct result of the CpG alone.

One of the main criticisms on this project was the choice of animal model for our studies. The B16-OVA expressing melanoma cells were designed to study the anti-tumor effects of molecules while expressing OVA as an antigen (Brown et al., 2001). To say the least, this kind of tumor is an optimal one to study in that it has high expression of the antigen on its surface. This is not usually the case, and in fact tumor cells can actually down regulate their MHC expression to avoid detection (as reviewed in (Algarra et al., 2004)). To fully establish our microparticle based vaccine's functionality, it would be better to test it in a more realistic tumor model with an actual tumor protein antigen.

Further, to show our particles' platform functionality, it may be useful to investigate its application to other disease models. We know that the hepatitis b surface antigen's isoelectric point is very close to that of ovalbumin's so transitioning into that disease model should be fairly simple. We conclude that given our past studies and planned studies we have an effective prototype set of particles that may be used in a safe, synthetically based vaccine system.

6.2 REFERENCES

Algarra, I., García-Lora, A., Cabrera, T., Ruiz-Cabello, F., and Garrido, F. (2004). The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol. Immunother. 53, 904–910.

Alves, C.M., Yang, Y., Carnes, D.L., Ong, J.L., Sylvia, V.L., Dean, D.D., Agrawal, C.M., and Reis, R.L. (2007). Modulating bone cells response onto starch-based biomaterials by surface plasma treatment and protein adsorption. Biomaterials 28, 307–315.

Alves, C.M., Yang, Y., Marton, D., Carnes, D.L., Ong, J.L., Sylvia, V.L., Dean, D.D., Reis, R.L., and Agrawal, C.M. (2008). Plasma surface modification of poly(D,L-lactic acid) as a tool to enhance protein adsorption and the attachment of different cell types. J. Biomed. Mater. Res. B Appl. Biomater. 87B, 59–66.

Armentano, I., Ciapetti, G., Pennacchi, M., Dottori, M., Devescovi, V., Granchi, D., Baldini, N., Olalde, B., Jurado, M.J., Alava, J.I.M., et al. (2009). Role of PLLA plasma surface modification in the interaction with human marrow stromal cells. J. Appl. Polym. Sci. 114, 3602–3611.

Bélard, L., Poncin-Epaillard, F., Dole, P., and Avérous, L. (2013). Plasma-polymer coatings onto different biodegradable polyesters surfaces. Eur. Polym. J. 49, 882–892.

Brown, D.M., Fisher, T.L., Wei, C., Frelinger, J.G., and Lord, E.M. (2001). Tumours can act as adjuvants for humoral immunity. Immunology 102, 486–497.

Cui, Z., and Qiu, F. (2006). Synthetic double-stranded RNA poly(I:C) as a potent peptide vaccine adjuvant: therapeutic activity against human cervical cancer in a rodent model. Cancer Immunol. Immunother. Cii 55, 1267–1279.

Desmet, T., Morent, R., De Geyter, N., Leys, C., Schacht, E., and Dubruel, P. (2009). Nonthermal plasma technology as a versatile strategy for polymeric biomaterials surface modification: a review. Biomacromolecules 10, 2351–2378.

Eisenbrey, J.R., Hsu, J., and Wheatley, M.A. (2009). Plasma Sterilization of Poly Lactic Acid Ultrasound Contrast Agents: Surface Modification and Implications for Drug Delivery. Ultrasound Med. Biol. 35, 1854–1862.

Hasirci, N., Endogan, T., Vardar, E., Kiziltay, A., and Hasirci, V. (2010). Effect of oxygen plasma on surface properties and biocompatibility of PLGA films. Surf. Interface Anal. 42, 486–491.

Heckelsmiller, K., Rall, K., Beck, S., Schlamp, A., Seiderer, J., Jahrsdörfer, B., Krug, A., Rothenfusser, S., Endres, S., and Hartmann, G. (2002). Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure

established tumors in a murine colon carcinoma model. J. Immunol. Baltim. Md 1950 169, 3892–3899.

Holy, C.E., Cheng, C., Davies, J.E., and Shoichet, M.S. (2001). Optimizing the sterilization of PLGA scaffolds for use in tissue engineering. Biomaterials 22, 25–31.

Kasturi, S.P., Skountzou, I., Albrecht, R.A., Koutsonanos, D., Hua, T., Nakaya, H.I., Ravindran, R., Stewart, S., Alam, M., Kwissa, M., et al. (2011). Programming the magnitude and persistence of antibody responses with innate immunity. Nature 470, 543–547.

Kigasawa, K., Kajimoto, K., Nakamura, T., Hama, S., Kanamura, K., Harashima, H., and Kogure, K. (2011). Noninvasive and efficient transdermal delivery of CpG-oligodeoxynucleotide for cancer immunotherapy. J. Controlled Release 150, 256–265.

Li, X., Sloat, B.R., Yanasarn, N., and Cui, Z. (2011). Relationship between the size of nanoparticles and their adjuvant activity: Data from a study with an improved experimental design. Eur. J. Pharm. Biopharm. 78, 107–116.

Li, Z., Beck, P., Ohlberg, D.A.., Stewart, D.R., and Williams, R.S. (2003). Surface properties of platinum thin films as a function of plasma treatment conditions. Surf. Sci. 529, 410–418.

Morent, R., De Geyter, N., Leys, C., Gengembre, L., and Payen, E. (2007). Study of the ageing behaviour of polymer films treated with a dielectric barrier discharge in air, helium and argon at medium pressure. Surf. Coatings Technol. 201, 7847–7854.

Shin, J., and Raja, L.L. (2007). Run-to-run variations, asymmetric pulses, and long time-scale transient phenomena in dielectric-barrier atmospheric pressure glow discharges. J. Phys. Appl. Phys. 40, 3145.

Vesel, A. (2010). Modification of polystyrene with a highly reactive cold oxygen plasma. Surf. Coatings Technol. 205, 490–497.

Vesel, A., and Mozetic, M. (2012). Surface modification and ageing of PMMA polymer by oxygen plasma treatment. Vacuum 86, 634–637.

Wan, Y., Qu, X., Lu, J., Zhu, C., Wan, L., Yang, J., Bei, J., and Wang, S. (2004). Characterization of surface property of poly(lactide-co-glycolide) after oxygen plasma treatment. Biomaterials 25, 4777–4783.

Bibliography

Aichele, P., Brduscha-Riem, K., Zinkernagel, R.M., Hengartner, H., and Pircher, H. (1995). T cell priming versus T cell tolerance induced by synthetic peptides. J. Exp. Med. *182*, 261–266.

Akasaki, Y., Kikuchi, T., Irie, M., Yamamoto, Y., Arai, T., Tanaka, T., Joki, T., and Abe, T. (2011). Cotransfection of Poly(I. J. Immunother. *0*, 121–128.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nat. Rev. Immunol. *4*, 499–511.

Algarra, I., García-Lora, A., Cabrera, T., Ruiz-Cabello, F., and Garrido, F. (2004). The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol. Immunother. *53*, 904–910.

Ali, O.A., Huebsch, N., Cao, L., Dranoff, G., and Mooney, D.J. (2009). Infection-mimicking materials to program dendritic cells in situ. Nat. Mater. *8*, 151–158.

Allard, S.D., De Keersmaecker, B., de Goede, A.L., Verschuren, E.J., Koetsveld, J., Reedijk, M.L., Wylock, C., De Bel, A.V., Vandeloo, J., Pistoor, F., et al. (2012). A phase I/IIa immunotherapy trial of HIV-1-infected patients with Tat, Rev and Nef expressing dendritic cells followed by treatment interruption. Clin. Immunol. *142*, 252–268.

Almeida, A.J., Alpar, H.O., and Brown, M.R.W. (1993). Immune Response to Nasal Delivery of Antigenically Intact Tetanus Toxoid Associated with Poly(l-lactic acid) Microspheres in Rats, Rabbits and Guinea-pigs. J. Pharm. Pharmacol. *45*, 198–203.

Alves, C.M., Yang, Y., Carnes, D.L., Ong, J.L., Sylvia, V.L., Dean, D.D., Agrawal, C.M., and Reis, R.L. (2007). Modulating bone cells response onto starch-based biomaterials by surface plasma treatment and protein adsorption. Biomaterials *28*, 307–315.

Alves, C.M., Yang, Y., Marton, D., Carnes, D.L., Ong, J.L., Sylvia, V.L., Dean, D.D., Reis, R.L., and Agrawal, C.M. (2008). Plasma surface modification of poly(D,L-lactic acid) as a tool to enhance protein adsorption and the attachment of different cell types. J. Biomed. Mater. Res. B Appl. Biomater. *87B*, 59–66.

American Cancer Society (2013). Cancer Facts & Figures 2013.

Armentano, I., Ciapetti, G., Pennacchi, M., Dottori, M., Devescovi, V., Granchi, D., Baldini, N., Olalde, B., Jurado, M.J., Alava, J.I.M., et al. (2009). Role of PLLA plasma

surface modification in the interaction with human marrow stromal cells. J. Appl. Polym. Sci. *114*, 3602–3611.

Arnon, R., and Horwitz, R.J. (1992). Synthetic peptides as vaccines. Curr. Opin. Immunol. *4*, 449–453.

Ataman-Önal, Y., Ataman-&Ouml, nal, Y., Munier, S., verine, Gan&eacute, e, A., Terrat, C., line, Durand, P.-Y., et al. (2006). Surfactant-free anionic PLA nanoparticles coated with HIV-1 p24 protein induced enhanced cellular and humoral immune responses in various animal models. J. Controlled Release *112*, 175–185.

Ataman-Önal, Y., Munier, S., Ganée, A., Terrat, C., Durand, P.-Y., Battail, N., Martinon, F., Le Grand, R., Charles, M.-H., Delair, T., et al. (2006). Surfactant-free anionic PLA nanoparticles coated with HIV-1 p24 protein induced enhanced cellular and humoral immune responses in various animal models. J. Controlled Release *112*, 175–185.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245–252.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.-J., Pulendran, B., and Palucka, K. (2000). Immunobiology of Dendritic Cells. Annu. Rev. Immunol. *18*, 767–811.

Basarkar, A., Devineni, D., Palaniappan, R., and Singh, J. (2007). Preparation, characterization, cytotoxicity and transfection efficiency of poly(dl-lactide-co-glycolide) and poly(dl-lactic acid) cationic nanoparticles for controlled delivery of plasmid DNA. Int. J. Pharm. *343*, 247–254.

Bélard, L., Poncin-Epaillard, F., Dole, P., and Avérous, L. (2013). Plasma-polymer coatings onto different biodegradable polyesters surfaces. Eur. Polym. J. *49*, 882–892.

Bharali, D.J., Pradhan, V., Elkin, G., Qi, W., Hutson, A., Mousa, S.A., and Thanavala, Y. (2008). Novel nanoparticles for the delivery of recombinant hepatitis B vaccine. Nanomedicine Nanotechnol. Biol. Med. *4*, 311–317.

Brown, D.M., Fisher, T.L., Wei, C., Frelinger, J.G., and Lord, E.M. (2001). Tumours can act as adjuvants for humoral immunity. Immunology *102*, 486–497.

Van der Bruggen, P., Stroobant, V., Vigneron, N., and Van den Eynde, B. (2013). Peptide database: T cell-defined tumor antigens.

Cai, C., Bakowsky, U., Rytting, E., Schaper, A.K., and Kissel, T. (2008). Charged nanoparticles as protein delivery systems: A feasibility study using lysozyme as model protein. Eur. J. Pharm. Biopharm. *69*, 31–42.

Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature *388*, 782–787.

Celluzzi, C.M., Mayordomo, J.I., Storkus, W.J., Lotze, M.T., and Falo, L.D. (1996). Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. J. Exp. Med. *183*, 283–287.

Chen, H., Li, P., Yin, Y., Cai, X., Huang, Z., Chen, J., Dong, L., and Zhang, J. (2010). The promotion of type 1 T helper cell responses to cationic polymers in vivo via toll-like receptor-4 mediated IL-12 secretion. Biomaterials *31*, 8172–8180.

Chollet, P., Favrot, M.C., Hurbin, A., and Coll, J.-L. (2002). Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. J. Gene Med. *4*, 84–91.

Cole, D.K., Edwards, E.S.J., Wynn, K.K., Clement, M., Miles, J.J., Ladell, K., Ekeruche, J., Gostick, E., Adams, K.J., Skowera, A., et al. (2010). Modification of MHC Anchor Residues Generates Heteroclitic Peptides That Alter TCR Binding and T Cell Recognition. J. Immunol. *185*, 2600–2610.

Cui, Z., and Mumper, R.J. (2002). Coating of cationized protein on engineered nanoparticles results in enhanced immune responses. Int. J. Pharm. *238*, 229–239.

Cui, Z., and Qiu, F. (2006). Synthetic double-stranded RNA poly(I:C) as a potent peptide vaccine adjuvant: therapeutic activity against human cervical cancer in a rodent model. Cancer Immunol. Immunother. Cii *55*, 1267–1279.

Cui, Z., Patel, J., Tuzova, M., Ray, P., Phillips, R., Woodward, J.G., Nath, A., and Mumper, R.J. (2004). Strong T cell type-1 immune responses to HIV-1 Tat (1–72) protein-coated nanoparticles. Vaccine *22*, 2631–2640.

Davis, H.L., Weeranta, R., Waldschmidt, T.J., Tygrett, L., Schorr, J., and Krieg, A.M. (1998). CpG DNA Is a Potent Enhancer of Specific Immunity in Mice Immunized with Recombinant Hepatitis B Surface Antigen. J. Immunol. *160*, 870–876.

Denis-Mize, K.S., Dupuis, M., MacKichan, M.L., Singh, M., Doe, B., O'Hagan, D., Ulmer, J.B., Donnelly, J.J., McDonald, D.M., and Ott, G. (2000). Plasmid DNA adsorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells. Gene Ther. *7*, 2105–2112.

Desmet, T., Morent, R., De Geyter, N., Leys, C., Schacht, E., and Dubruel, P. (2009). Nonthermal plasma technology as a versatile strategy for polymeric biomaterials surface modification: a review. Biomacromolecules *10*, 2351–2378.

Dhodapkar, M.V., Steinman, R.M., Krasovsky, J., Munz, C., and Bhardwaj, N. (2001). Antigen-Specific Inhibition of Effector T Cell Function in Humans after Injection of Immature Dendritic Cells. J. Exp. Med. *193*, 233–238.

Diwan, M., Tafaghodi, M., and Samuel, J. (2002). Enhancement of immune responses by co-delivery of a CpG oligodeoxynucleotide and tetanus toxoid in biodegradable nanospheres. J. Controlled Release *85*, 247–262.

Djordjevic, I., Britcher, L.G., and Kumar, S. (2008). Morphological and surface compositional changes in poly(lactide-co-glycolide) tissue engineering scaffolds upon radio frequency glow discharge plasma treatment. Appl. Surf. Sci. *254*, 1929–1935.

Ehlers, S., and Bulfone-Paus, S. (2004). Shaping Adaptive Immunity against Pathogens: The Contribution of Innate Immune Responses. In Novel Vaccination Strategies, S.H.E. Kaufmann, ed. (Wiley-VCH Verlag GmbH & Co. KGaA), pp. 17–50.

Eisenbrey, J.R., Hsu, J., and Wheatley, M.A. (2009). Plasma Sterilization of Poly Lactic Acid Ultrasound Contrast Agents: Surface Modification and Implications for Drug Delivery. Ultrasound Med. Biol. *35*, 1854–1862.

Euhus, D.M., Hudd, C., Laregina, M.C., and Johnson, F.E. (1986). Tumor measurement in the nude mouse. J. Surg. Oncol. *31*, 229–234.

Fischer, S., Uetz-von Allmen, E., Waeckerle-Men, Y., Groettrup, M., Merkle, H.P., and Gander, B. (2007). The preservation of phenotype and functionality of dendritic cells upon phagocytosis of polyelectrolyte-coated PLGA microparticles. Biomaterials *28*, 994–1004.

Foged, C., Brodin, B., Frokjaer, S., and Sundblad, A. (2005). Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model. Int. J. Pharm. *298*, 315–322.

Fortunati, E., Mattioli, S., Visai, L., Imbriani, M., Fierro, J.L.G., Kenny, J.M., and Armentano, I. (2013). Combined Effects of Ag Nanoparticles and Oxygen Plasma Treatment on PLGA Morphological, Chemical, and Antibacterial Properties. Biomacromolecules.

Friede, M., and Aguado, M.T. (2005). Need for new vaccine formulations and potential of particulate antigen and DNA delivery systems. Adv. Drug Deliv. Rev. *57*, 325–331.

García, F., and Routy, J.-P. (2011). Challenges in dendritic cells-based therapeutic vaccination in HIV-1 infection: Workshop in dendritic cell-based vaccine clinical trials in HIV-1. Vaccine *29*, 6454–6463.

García, F., Climent, N., Guardo, A.C., Gil, C., León, A., Autran, B., Lifson, J.D., Martínez-Picado, J., Dalmau, J., Clotet, B., et al. (2013). A Dendritic Cell–Based Vaccine Elicits T Cell Responses Associated with Control of HIV-1 Replication. Sci. Transl. Med. *5*, 166ra2–166ra2.

Godbey, W.T., Wu, K.K., and Mikos, A.G. (1999). Poly(ethylenimine) and its role in gene delivery. J. Controlled Release *60*, 149–160.

Guy, B. (2007). The perfect mix: recent progress in adjuvant research. Nat. Rev. Microbiol. *5*, 505–517.

Hailemichael, Y., Dai, Z., Jaffarzad, N., Ye, Y., Medina, M.A., Huang, X.-F., Dorta-Estremera, S.M., Greeley, N.R., Nitti, G., Peng, W., et al. (2013). Persistent antigen at vaccination sites induces tumor-specific CD8+ T cell sequestration, dysfunction and deletion. Nat. Med. *19*, 465–472.

Halperin, S.A., Van Nest, G., Smith, B., Abtahi, S., Whiley, H., and Eiden, J.J. (2003). A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. Vaccine *21*, 2461–2467.

Han, R., Zhu, J., Yang, X., and Xu, H. (2011). Surface modification of poly(D,L-lactic-co-glycolic acid) nanoparticles with protamine enhanced cross-presentation of encapsulated ovalbumin by bone marrow-derived dendritic cells. J. Biomed. Mater. Res. A *96A*, 142–149.

Hartmann, G., Weiner, G.J., and Krieg, A.M. (1999). CpG DNA: A potent signal for growth, activation, and maturation of human dendritic cells. Proc. Natl. Acad. Sci. *96*, 9305–9310.

Hasirci, N., Endogan, T., Vardar, E., Kiziltay, A., and Hasirci, V. (2010). Effect of oxygen plasma on surface properties and biocompatibility of PLGA films. Surf. Interface Anal. *42*, 486–491.

Heckelsmiller, K., Rall, K., Beck, S., Schlamp, A., Seiderer, J., Jahrsdörfer, B., Krug, A., Rothenfusser, S., Endres, S., and Hartmann, G. (2002). Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. J. Immunol. Baltim. Md 1950 *169*, 3892–3899.

Hermans, I.F., Silk, J.D., Gileadi, U., Masri, S.H., Shepherd, D., Farrand, K.J., Salio, M., and Cerundolo, V. (2007). Dendritic Cell Function Can Be Modulated through Cooperative Actions of TLR Ligands and Invariant NKT Cells. J. Immunol. *178*, 2721–2729.

Holy, C.E., Cheng, C., Davies, J.E., and Shoichet, M.S. (2001). Optimizing the sterilization of PLGA scaffolds for use in tissue engineering. Biomaterials *22*, 25–31.

Huang, Y.-C., Huang, C.-C., Huang, Y.-Y., and Chen, K.-S. (2007). Surface modification and characterization of chitosan or PLGA membrane with laminin by chemical and oxygen plasma treatment for neural regeneration. J. Biomed. Mater. Res. A *82A*, 842–851.

Hubbell, J.A., Thomas, S.N., and Swartz, M.A. (2009). Materials engineering for immunomodulation. Nature *462*, 449–460.

Ikada, Y. (1994). Surface modification of polymers for medical applications. Biomaterials *15*, 725–736.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. *176*, 1693–1702.

Jensen, M.M., Jørgensen, J.T., Binderup, T., and Kjær, A. (2008). Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper. Bmc Med. Imaging *8*, 16.

Jiang, W., Gupta, R.K., Deshpande, M.C., and Schwendeman, S.P. (2005). Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. Adv. Drug Deliv. Rev. *57*, 391–410.

Jung, T., Kamm, W., Breitenbach, A., Hungerer, K.-D., Hundt, E., and Kissel, T. (2001). Tetanus Toxoid Loaded Nanoparticles from Sulfobutylated Poly(Vinyl Alcohol)-Graft-Poly(Lactide-co-Glycolide): Evaluation of Antibody Response After Oral and Nasal Application in Mice. Pharm. Res. *18*, 352–360.

Kantoff, P.W., Higano, C.S., Shore, N.D., Berger, E.R., Small, E.J., Penson, D.F., Redfern, C.H., Ferrari, A.C., Dreicer, R., Sims, R.B., et al. (2010). Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer. N. Engl. J. Med. *363*, 411–422.

Kasturi, S.P., Sachaphibulkij, K., and Roy, K. (2005). Covalent conjugation of polyethyleneimine on biodegradable microparticles for delivery of plasmid DNA vaccines. Biomaterials *26*, 6375–6385.

Kasturi, S.P., Skountzou, I., Albrecht, R.A., Koutsonanos, D., Hua, T., Nakaya, H.I., Ravindran, R., Stewart, S., Alam, M., Kwissa, M., et al. (2011). Programming the magnitude and persistence of antibody responses with innate immunity. Nature *470*, 543–547.

Kazzaz, J., Neidleman, J., Singh, M., Ott, G., and O'Hagan, D.T. (2000). Novel anionic microparticles are a potent adjuvant for the induction of cytotoxic T lymphocytes against recombinant p55 gag from HIV-1. J. Control. Release Off. J. Control. Release Soc. *67*, 347–356.

Kigasawa, K., Kajimoto, K., Nakamura, T., Hama, S., Kanamura, K., Harashima, H., and Kogure, K. (2011). Noninvasive and efficient transdermal delivery of CpG-oligodeoxynucleotide for cancer immunotherapy. J. Controlled Release *150*, 256–265.

Klebanoff, C.A., Acquavella, N., Yu, Z., and Restifo, N.P. (2011). Therapeutic cancer vaccines: are we there yet? Immunol. Rev. *239*, 27–44.

Klinman, D.M., Barnhart, K.M., and Conover, J. (1999). CpG motifs as immune adjuvants. Vaccine *17*, 19–25.

Kool, M., Fierens, K., and Lambrecht, B.N. (2011). Alum adjuvant: some of the tricks of the oldest adjuvant. J. Med. Microbiol. *61*, 927–934.

Krieg, A.M. (2002). Cpg Motifs in Bacterial Dna and Their Immune Effects*. Annu. Rev. Immunol. *20*, 709–760.

Lamalle-Bernard, D., Munier, S., Compagnon, C., Charles, M.-H., Kalyanaraman, V.S., Delair, T., Verrier, B., and Ataman-Önal, Y. (2006). Coadsorption of HIV-1 p24 and gp120 proteins to surfactant-free anionic PLA nanoparticles preserves antigenicity and immunogenicity. J. Controlled Release *115*, 57–67.

Laroussi, M. (2005). Low Temperature Plasma-Based Sterilization: Overview and State-of-the-Art. Plasma Process. Polym. *2*, 391–400.

Lee, S.G., An, E. young, Lee, J.B., Park, J.C., Shin, J.W., and Kim, J.K. (2007). Enhanced cell affinity of poly(d,l-lactic-co-glycolic acid) (50/50) by plasma treatment with β-(1 → 3) (1 → 6)-glucan. Surf. Coatings Technol. *201*, 5128–5131.

Leleux, J., and Roy, K. (2013a). Micro and Nanoparticle-Based Delivery Systems for Vaccine Immunotherapy: An Immunological and Materials Perspective. Adv. Healthc. Mater. *2*, 72–94.

Leleux, J., and Roy, K. (2013b). Micro and Nanoparticle-Based Delivery Systems for Vaccine Immunotherapy: An Immunological and Materials Perspective. Adv. Healthc. Mater. *2*, 72–94.

Li, X., Sloat, B.R., Yanasarn, N., and Cui, Z. (2011a). Relationship between the size of nanoparticles and their adjuvant activity: Data from a study with an improved experimental design. Eur. J. Pharm. Biopharm. *78*, 107–116.

Li, X., Sloat, B.R., Yanasarn, N., and Cui, Z. (2011b). Relationship between the size of nanoparticles and their adjuvant activity: Data from a study with an improved experimental design. Eur. J. Pharm. Biopharm. *78*, 107–116.

Li, Z., Beck, P., Ohlberg, D.A.., Stewart, D.R., and Williams, R.S. (2003). Surface properties of platinum thin films as a function of plasma treatment conditions. Surf. Sci. *529*, 410–418.

Lipford, G.B., Bauer, M., Blank, C., Reiter, R., Wagner, H., and Heeg, K. (1997). CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. Eur. J. Immunol. *27*, 2340–2344.

Liu, G., Ng, H., Akasaki, Y., Yuan, X., Ehtesham, M., Yin, D., Black, K.L., and Yu, J.S. (2004). Small interference RNA modulation of IL-10 in human monocyte-derived dendritic cells enhances the Th1 response. Eur. J. Immunol. *34*, 1680–1687.

Lutz, M.B., and Schuler, G. (2002). Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol. *23*, 445–449.

Mahnke, K., Schmitt, E., Bonifaz, L., Enk, A.H., and Jonuleit, H. (2002). Immature, but not inactive: the tolerogenic function of immature dendritic cells. Immunol. Cell Biol. *80*, 477–483.

Malyala, P., Chesko, J., Ugozzoli, M., Goodsell, A., Zhou, F., Vajdy, M., O'Hagan, D.T., and Singh, M. (2008). The potency of the adjuvant, CpG oligos, is enhanced by encapsulation in PLG microparticles. J. Pharm. Sci. *97*, 1155–1164.

Marques, J.T., and Williams, B.R.G. (2005). Activation of the mammalian immune system by siRNAs. Nat. Biotechnol. *23*, 1399–1405.

Mattioli, S., Kenny, J.M., and Armentano, I. (2012). Plasma surface modification of porous PLLA films: Analysis of surface properties and in vitro hydrolytic degradation. J. Appl. Polym. Sci. *125*, E239–E247.

Milacic, V., Bailey, B.A., O'Hagan, D., and Schwendeman, S.P. (2012). Injectable PLGA Systems for Delivery of Vaccine Antigens. In Long Acting Injections and Implants, J.C. Wright, and D.J. Burgess, eds. (Springer US), pp. 429–458.

Morent, R., De Geyter, N., Leys, C., Gengembre, L., and Payen, E. (2007). Study of the ageing behaviour of polymer films treated with a dielectric barrier discharge in air, helium and argon at medium pressure. Surf. Coatings Technol. *201*, 7847–7854.

Morent, R., Geyter, N., Trentesaux, M., Gengembre, L., Dubruel, P., Leys, C., and Payen, E. (2010). Influence of Discharge Atmosphere on the Ageing Behaviour of Plasma-Treated Polylactic Acid. Plasma Chem. Plasma Process. *30*, 525–536.

Morent, R., De Geyter, N., Desmet, T., Dubruel, P., and Leys, C. (2011). Plasma Surface Modification of Biodegradable Polymers: A Review. Plasma Process. Polym. *8*, 171–190.

Mosca, F., Tritto, E., Muzzi, A., Monaci, E., Bagnoli, F., Iavarone, C., O'Hagan, D., Rappuoli, R., and Gregorio, E.D. (2008). Molecular and cellular signatures of human vaccine adjuvants. Proc. Natl. Acad. Sci. *105*, 10501–10506.

Nicholson, K.G., Colegate, A.E., Podda, A., Stephenson, I., Wood, J., Ypma, E., and Zambon, M.C. (2001). Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. The Lancet *357*, 1937–1943.

O'Hagan, D.T., and Singh, M. (2003). Microparticles as vaccine adjuvants and delivery systems. Expert Rev. Vaccines *2*, 269–283.

O'Hagan, D.T., Singh, M., and Ulmer, J.B. (2004). Microparticles for the delivery of DNA vaccines. Immunol. Rev. *199*, 191–200.

Pai Kasturi, S., Qin, H., Thomson, K.S., El-Bereir, S., Cha, S.-C., Neelapu, S., Kwak, L.W., and Roy, K. (2006). Prophylactic anti-tumor effects in a B cell lymphoma model with DNA vaccines delivered on polyethylenimine (PEI) functionalized PLGA microparticles. J. Control. Release Off. J. Control. Release Soc. *113*, 261–270.

Palucka, K., and Banchereau, J. (2012). Cancer immunotherapy via dendritic cells. Nat. Rev. Cancer *12*, 265–277.

Park, J., Wu, C.T., and Bryers, J.D. (2013). Chemokine programming dendritic cell antigen response: part I - select chemokine programming of antigen uptake even after maturation. Immunology *139*, 72–87.

Perrie, Y., Mohammed, A.R., Kirby, D.J., McNeil, S.E., and Bramwell, V.W. (2008). Vaccine adjuvant systems: Enhancing the efficacy of sub-unit protein antigens. Int. J. Pharm. *364*, 272–280.

Pierre, P., Turley, S., Gatti, E., Hull, M., Meltzer, J., Mizra, A., Inaba, K., Steinman, R., and Mellman, I. (1997). Developmental regulation of MHC class II transport in mouse dendritic cells. Nature *388*, 787–792.

Plotkin, S. (2003). Disease States and Vaccines: Selected Cases. In The Vaccine Book, (San Diego, California: Academic Press), pp. 179–188.

Predina, J., Eruslanov, E., Judy, B., Kapoor, V., Cheng, G., Wang, L.-C., Sun, J., Moon, E.K., Fridlender, Z.G., Albelda, S., et al. (2013). Changes in the local tumor

microenvironment in recurrent cancers may explain the failure of vaccines after surgery. Proc. Natl. Acad. Sci. *110*, E415–E424.

Pulendran, B., Palucka, K., and Banchereau, J. (2001). Sensing Pathogens and Tuning Immune Responses. Science *293*, 253–256.

Purcell, A.W., McCluskey, J., and Rossjohn, J. (2007). More than one reason to rethink the use of peptides in vaccine design. Nat. Rev. Drug Discov. *6*, 404–414.

Rosenberg, S.A., Yang, J.C., and Restifo, N.P. (2004). Cancer immunotherapy: moving beyond current vaccines. Nat. Med. *10*, 909–915.

Rosenberg, S.A., Yang, J.C., Kammula, U.S., Hughes, M.S., Restifo, N.P., Schwarz, S.L., Morton, K.E., Laurencot, C.M., and Sherry, R.M. (2010). Different Adjuvanticity of Incomplete Freund's Adjuvant Derived From Beef or Vegetable Components in Melanoma Patients Immunized With a Peptide Vaccine. J. Immunother. *33*, 626–629.

Rutella, S., Danese, S., and Leone, G. (2006). Tolerogenic dendritic cells: cytokine modulation comes of age. Blood *108*, 1435–1440.

Salvador, A., Igartua, M., Hernández, R.M., and Pedraz, J.L. (2012). Combination of immune stimulating adjuvants with poly(lactide-co-glycolide) microspheres enhances the immune response of vaccines. Vaccine *30*, 589–596.

Sato, M., Iwakabe, K., Kimura, S., and Nishimura, T. (1999). Functional skewing of bone marrow-derived dendritic cells by Th1- or Th2-inducing cytokines. Immunol. Lett. *67*, 63–68.

Sette, A., and Fikes, J. (2003). Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. Curr. Opin. Immunol. *15*, 461–470.

Shau, M.D., Shih, M.F., Lin, C.C., Chuang, I.C., Hung, W.C., Hennink, W.E., and Cherng, J.Y. (2012). A one-step process in preparation of cationic nanoparticles with poly(lactide-co-glycolide)-containing polyethylenimine gives efficient gene delivery. Eur. J. Pharm. Sci. *46*, 522–529.

Shen, H., Hu, X., Bei, J., and Wang, S. (2008). The immobilization of basic fibroblast growth factor on plasma-treated poly(lactide-co-glycolide). Biomaterials *29*, 2388–2399.

Shin, J., and Raja, L.L. (2003). Dynamics of pulse phenomena in helium dielectric-barrier atmospheric-pressure glow discharges. J. Appl. Phys. *94*, 7408–7415.

Shin, J., and Raja, L.L. (2007). Run-to-run variations, asymmetric pulses, and long time-scale transient phenomena in dielectric-barrier atmospheric pressure glow discharges. J. Phys. Appl. Phys. *40*, 3145.

Singh, A., Nie, H., Ghosn, B., Qin, H., Kwak, L.W., and Roy, K. (2008). Efficient Modulation of T-cell Response by Dual-mode, Single-carrier Delivery of Cytokine-targeted siRNA and DNA Vaccine to Antigen-presenting Cells. Mol. Ther. *16*, 2011–2021.

Singh, A., Suri, S., and Roy, K. (2009). In-situ crosslinking hydrogels for combinatorial delivery of chemokines and siRNA-DNA carrying microparticles to dendritic cells. Biomaterials *30*, 5187.

Singh, M., Briones, M., Ott, G., and O'Hagan, D. (2000). Cationic microparticles: A potent delivery system for DNA vaccines. Proc. Natl. Acad. Sci. *97*, 811–816.

Singh, M., Ott, G., Kazzaz, J., Ugozzoli, M., Briones, M., Donnelly, J., and O'Hagan, D.T. (2001). Cationic Microparticles Are an Effective Delivery System for Immune Stimulatory CpG DNA. Pharm. Res. *18*, 1476–1479.

Singh, M., Kazzaz, J., Chesko, J., Soenawan, E., Ugozzoli, M., Giuliani, M., Pizza, M., Rappouli, R., and O'Hagan, D.T. (2004a). Anionic microparticles are a potent delivery system for recombinant antigens from Neisseria meningitidis serotype B. J. Pharm. Sci. *93*, 273–282.

Singh, M., Chesko, J., Kazzaz, J., Ugozzoli, M., Kan, E., Srivastava, I., and O'Hagan, D.T. (2004b). Adsorption of a novel recombinant glycoprotein from HIV (Env gp120dV2 SF162) to anionic PLG microparticles retains the structural integrity of the protein, whereas encapsulation in PLG microparticles does not. Pharm. Res. *21*, 2148–2152.

Singh, M., Kazzaz, J., Ugozzoli, M., Chesko, J., and O'Hagan, D.T. (2004c). Charged polylactide co-glycolide microparticles as antigen delivery systems. Expert Opin. Biol. Ther. *4*, 483–491.

Siow, K.S., Britcher, L., Kumar, S., and Griesser, H.J. (2006). Plasma Methods for the Generation of Chemically Reactive Surfaces for Biomolecule Immobilization and Cell Colonization - A Review. Plasma Process. Polym. *3*, 392–418.

Soppimath, K.S., Aminabhavi, T.M., Kulkarni, A.R., and Rudzinski, W.E. (2001). Biodegradable polymeric nanoparticles as drug delivery devices. J. Controlled Release *70*, 1–20.

Steinman, R.M., and Pope, M. (2002). Exploiting dendritic cells to improve vaccine efficacy. J. Clin. Invest. *109*, 1519–1526.

Tendero, C., Tixier, C., Tristant, P., Desmaison, J., and Leprince, P. (2006). Atmospheric pressure plasmas: A review. Spectrochim. Acta Part B At. Spectrosc. *61*, 2–30.

Thompson, C.B. (1995). Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation? Cell *81*, 979–982.

Tomayko, M.M., and Reynolds, C.P. (1989). Determination of subcutaneous tumor size in athymic (nude) mice. Cancer Chemother. Pharmacol. *24*, 148–154.

Trollfors, B., Bergfors, E., and Inerot, A. (2005). Vaccine related itching nodules and hypersensitivity to aluminium. Vaccine *23*, 975–976.

Vajdy, M., Srivastava, I., Polo, J., Donnelly, J., O'Hagan, D., and Singh, M. (2004). Mucosal adjuvants and delivery systems for protein-, DNA- and RNA-based vaccines. Immunol. Cell Biol. *82*, 617–627.

Vesel, A. (2010). Modification of polystyrene with a highly reactive cold oxygen plasma. Surf. Coatings Technol. *205*, 490–497.

Vesel, A., and Mozetic, M. (2012). Surface modification and ageing of PMMA polymer by oxygen plasma treatment. Vacuum *86*, 634–637.

Waeckerle-Men, Y., Scandella, E., Uetz-von Allmen, E., Ludewig, B., Gillessen, S., Merkle, H.P., Gander, B., and Groettrup, M. (2004). Phenotype and functional analysis of human monocyte-derived dendritic cells loaded with biodegradable poly(lactide-co-glycolide) microspheres for immunotherapy. J. Immunol. Methods *287*, 109–124.

Wan, Y., Qu, X., Lu, J., Zhu, C., Wan, L., Yang, J., Bei, J., and Wang, S. (2004). Characterization of surface property of poly(lactide-co-glycolide) after oxygen plasma treatment. Biomaterials *25*, 4777–4783.

Wang, J., Feng, S.-S., Wang, S., and Chen, Z. (2010). Evaluation of cationic nanoparticles of biodegradable copolymers as siRNA delivery system for hepatitis B treatment. Int. J. Pharm. *400*, 194–200.

Weert, M. van de, Hennink, W.E., and Jiskoot, W. (2000). Protein Instability in Poly(Lactic-co-Glycolic Acid) Microparticles. Pharm. Res. *17*, 1159–1167.

Wischke, C., Borchert, H.-H., Zimmermann, J., Siebenbrodt, I., and Lorenzen, D.R. (2006). Stable cationic microparticles for enhanced model antigen delivery to dendritic cells. J. Control. Release Off. J. Control. Release Soc. *114*, 359–368.

Xie, Hang, Gursel, Ihsan, Ivins, Bruce E, Singh, Monmohan, O'Hagan, Derek T, Ulmer, J.B., and Klinman, D.M. (2005). CpG Oligodeoxynucleotides Adsorbed onto Polylactide-Co-Glycolide Microparticles Improve the Immunogenicity and Protective Activity of the Licensed Anthrax Vaccine. Infect. Immun. *73*, 828–833.

Ye, M., Kim, S., and Park, K. (2010). Issues in long-term protein delivery using biodegradable microparticles. J. Controlled Release *146*, 241–260.

Ben-Yedidia, T., and Arnon, R. (1997). Design of peptide and polypeptide vaccines. Curr. Opin. Biotechnol. *8*, 442–448.

Zwiorek, K., Bourquin, C., Battiany, J., Winter, G., Endres, S., Hartmann, G., and Coester, C. (2008). Delivery by Cationic Gelatin Nanoparticles Strongly Increases the Immunostimulatory Effects of CpG Oligonucleotides. Pharm. Res. *25*, 551–562.